Bleaching of Melanomacrophages from Tissues of Ectothermic Vertebrates for Later Use of Immunohistochemical and in Situ Hybridization Technique

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Abstract— *Due to the large quantity of melanomacrophages in the organs of the ectothermic vertebrates, with special interest in the ranids and fish, with their brownish melanin granules, we decided to test the MELANIN removal technique, in order to facilitate the observation of the organ fragments in the slides, under the direct light optical microscope, when using the antibodies and biotinylated probes.*

Thus, the melanin bleaching study favored the visualization of the diaminobenzidine chromogen (DAB) without interfering with the antigen-antibody affinity of immunohistochemistry and without interfering with the technique by which specific nucleotide sequences are identified in histological sections. (of DNA or RNA, endogenous, bacterial or viral).

This bleaching of melanin from tissues avoided false positive results, without interfering with the IHQ and ISH techniques for Mycobacterium spp and Francisella spp in fish.

Keywords— Bleaching, ectothermic vertebrates, IHQ and ISH techniques, melanomacrophages.

I. INTRODUCTION

Fish, especially teleosts as well as amphibians, share with the ectothermic vertebrates the existence of an extra-cutaneous pigment system consisting of large, irregular cells in various tissues and organs that can produce and storing melanin within them. This variable amount of melanomacrophages centers or macrophage aggregates are usually found on a larger scale within the endothelial reticulum in the hematopoietic matrix, in the spleen and kidney, but also in the liver (MESSEGUER et al., 1994) and to a lesser extent in the submucosa of the intestine, thymus, gills, brain and gonads of teleosteal fish. The emergence of these structures is related to several factors and conditions, such as organ, age, nutritional status, anatomopathological conditions, as well as environmental changes such as in the processes of detoxification by pollutants and cytoprotective functions related to free radicals.

In the liver, kidneys and spleen, the MMs may contain melanin, cellular fragments, hemosiderin granules and lipofucsine residues, mainly due to their functions of cellular debris sequestration and potentially toxic materials, in addition to participating in the response of fishes and ranids to infectious agents such as fungi and bacterias (STEINEL, BOLNICK, 2017)

The IHQ technique uses the enzyme peroxidase and DAB (3'-3-diaminobenzidine tetrahydrochloride) as the most common and inexpensive chromogen and can have its reaction visualized in a direct light optic microscope, allowing, for example, the diagnosis of various agents' infectious diseases. DAB, however, presents as a marker of deep brown color and may become indistinguishable from the brownish granules of melanin, causing diagnostic errors when viewed under the microscope.

The ISH reaction allows to accurately localizing in the paraffin or frozen tissue, a specific gene or its transcripts. The technique also allows to associate the presence of DNA or mRNA of microorganisms with the morphology, or even to relate the presence of genes and their transcripts with the pathological processes. Thus, there is the association with immunohistochemistry and the ribosondes can be used to study, for example, viral, bacterial, etc. infections, and to differentiate productive viral infections from non-productive ones.

The purpose of this work was to standardize a simple and efficient protocol to bleach the melanin in heavily pigmented tissues. The bleaching technique allowed a correct observation of the chromogen in the antibody-antigen (IHQ) reaction and in the ISH probes in the tissue, not interfering with their affinity and thus avoiding false positives.

II. MATERIAL AND METHODS

Fragments of fish organs collected between apparently healthy animals or diseased animals were fixed in 10% formalin, dehydrated in increasing sequence of alcohols and diaphanized in xylol. After embedded in paraffin, histological sections of 4.5 microns in thickness were glued on silanized slides. With each fragment 3 slides were made. Thus, in 1/3 of the slides the IHC technique was used to search for mycobacterium or francisella using organ fragments the same as the control fragment without adding the antibody. (protocol: blocking the endogenous peroxidase with 3% H_2O_2), the slides were washed with distilled water, followed by a bath with phosphate buffer (0.1 M PBS) at room temperature, 100 µl of the anti-mycobacterium and anti-francisella monoclonal antibodies (Synapse Biotechnology Ltd.) with Dako background reducer (Code S3022) were applied in each slide and incubated in a humid chamber for 18 hours in a refrigerator (2-8oC) .After this period, two washes were carried out with buffer. The system of visualization used was LSAB® + System-HRP (Dako-code K0690) with adaptation of the protocol suggested by the manufacturer. Incubation times of 20 minutes at the substrate-chromogen system was used as the Liquid DAB + Substrat Chromogen System (Dako - code K3468), incubated at room temperature for each of the reactants, intercalated with two washes with phosphate buffer (0.1M PBS) for 1 minute. for 5 minutes at room temperature, followed by washing in running distilled water. Counterstaining was done with hematoxylin).

The sequence of individual primers for *Francisella* spp. (Hsieh et al., 2007) was FLB16S180f: 5'-GCG-GATTAA-AGG-TGG-CCT (Talaat et al., 1997). Trim-C-3 '(forward primer) and FLB16S465r: 5'-CCT-GCA-AGC-TAT-TAA-CTC-ACAGG-3' (reverse primer) for *Mycobacterium* spp. which were specifically amplified: 924-bp fragments based on T-39 (5'G GCGAACGGGTGAGTAACACG-3') andT-13 (5'-TGCACACAGGCCACAAGGGA-3'). The antigen retrieval slides were pre-treated using a water bath at 40 ° C and buffer diluted for recovery (Dako S1699) for 40 min. When the slides are well cooled, the endogenous peroxidase is blocked at room temperature for 20 min and after enzymatic digestion of the tissues with proteinase k , Dako, at room temperature for 5-15 min. Specific biotinylated probes were mixed including the target DNA or RNA on the tissue and covering they with coverslip. Samples and probes were denatured and hybridized overnight (18 h) in the Dako hybridizer (denaturation at 96 ° C and hybridization at 37 ° C). After the stringency bath, TBST (Tris buffered saline / Tween) was washed. The visualization system used: primary streptoavidin in diluent buffer (Dako - kit code K0690) for 30 minutes in a humid chamber, Biotinil Tiramide reagent for 15 min at room temperature and then secondary streptoavidin for 15 min. All applications will be interspersed with two washes with TBST buffer for 5 minutes. The substrate-chromogen system used will be the Liquid DAB + Substrat Chromogen System (Dako - code K3468), incubated for 5 minutes at room temperature, followed by washing in running distilled water. The counterstaining was done with hematoxylin).

The slides used for the bleaching were immersed in 10% hydrogen peroxide (H_2O_2) in 0.2 mol / L Tris-HCl buffer pH 7.4 for 24 hours at room temperature. During this process, the material was kept in the dark. After this procedure, the normal staining protocol for IHC and ISH was followed.

III. RESULTS

Mycobacterium and Francisella positive animals were easily diagnosed using IHC and ISH techniques on slides in which the fragments were bleached with hydrogen peroxide as compared to those that were not cleared. (Figure 1, 2 and 3).

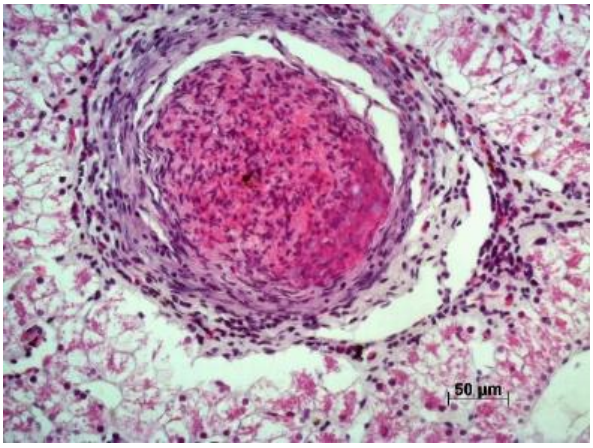

FIGURE 1: No melanin after treatment X200

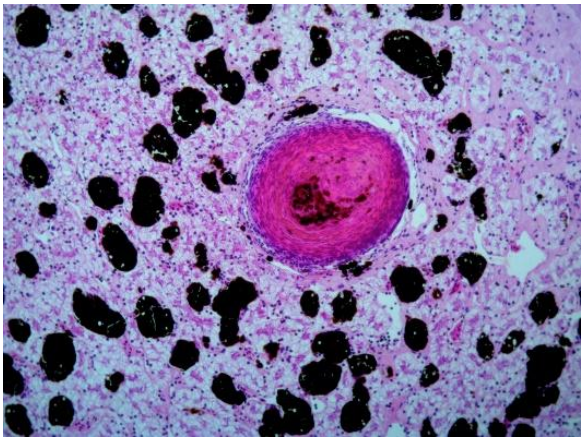

FIGURE 2: With melanin in the granuloma and in melanomacrophages X100

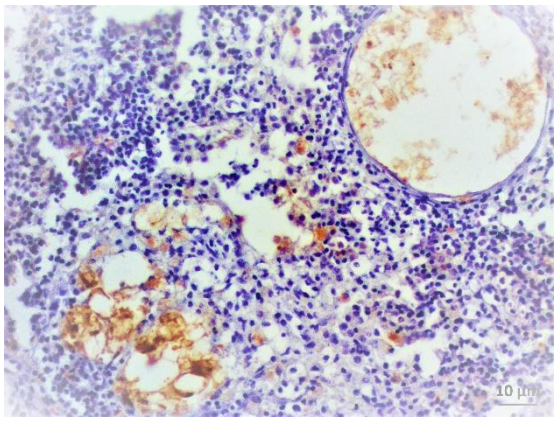

FIGURE 3: Photomicrograph of positive spleen for *Francisella spp*, after treatment, with the in-situ hybridization technique X 400

IV. DISCUSSION AND CONCLUSION

Among the cutaneous pigment macromolecules, we have melanin, eumelanin and pheomelanin (BILLINGHAM & SILVERS 1960, LING 1974). The extra-cutaneous melanin found in melanomacrophages tissues of ectothermic vertebrates is formed by the conversion of tyrosine into alpha-3, 4-dihydroxyphenylalanine and then into dopaquinone and melanin by tyrosinase (WOOLF & SWAFFORD, 1988). There are numerous functions attributed to melanomacrophages such as: antioxidant function, helping to protect the lipids of cell membranes from free radical attack (STEINEL, NC, BOLNICK, 2017), phagocytosis of resistant pathogens such as spores of parasites and bacterias (ROBERTS, 1975); processing of antigens in the immune response (AGIUS, 1985); destruction, detoxification or recycling of endogenous and exogenous materials (FERGUSON, 1976; ELLIS, 1980, HERRAEZ; ZAPATA, 1986); and the response to foreign bodies, including infectious agents (AGIUS, ROBERTS, 2003). Melanomacrophage centers are associated with the presence of acid-resistant intracellular bacteria such as mycobacteria and parasites such as *Myxobolus* spp. (ROBERTS, 2001). There is evidence that melanomacrophages play a relevant role in the control of myxosporidia infections (SUPAMATTAYA et al., 1993).

Bleaching is by gradual oxidation by hydrogen peroxide (KORYTWSKI & SARNA, 1990) and it was evident from our work that the visualization and analysis of IHQ and ISH for francisella and mycobacterium with DAB chromogen use will not be overestimated leading to false positives. This is especially interesting when doing computerized image analysis. These same results were obtained by SILVA, A P and collaborators, in 2011, using the melanin whitening in the epidermis of the South American sea lion for later application of enzymatic immunohistochemistry. It is concluded to be an effective whitening technique for any histological or histopathological study in any animal tissue with melanin.

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REFERENCES

[1] Steinel, N.C.; Bolnick ,D.I. **Melanomacrophage Centers As a Histological indicator of immune Function in Fish and Other Poikilotherms**. Front Immunol.; 8: 827,2017.

[2] Messeguer, J., Lopezruiz, A., Esteban, M., **Melanomacrophages of the seawater teleosts, sea bass (Dicentrarchus labrax) and gilthead seabream (Sparus aurata) – morphology, formation and possible function**. Cell Tiss. Res. 277, 1–10, 1994.

[3] Hsieh CY,Wu ZB, Tung MC, Tsai SS**. PCR and in situ hybridization for the detection and localization of a new pathogen Francisella-like bacterium (FLB) in ornamental cichlids.** Dis Aquat. Organ. 2007; 75:29–36, 2007.

[4] Talaat AM, Reimschuessel R, Trucksis M**. Identification of mycobacteria infecting fish to the species level using polymerase chain reaction and restriction enzyme analysis**. Vet. Micro., 58: 229-237,1997.

[5] Billingham R.E.; Silvers W.K. **The melanocytes of mammals**. Quart. Rev. Biology 35(1):1-40, 1960

[6] Ling J.K. **The integument of marine mammals**, p.1-42. In: Harrison R.J. (Ed.), Functional Anatomy of Marine Mammals. Vol.2. Academic Press, London.,1974

[7] Woolf C.M. & Swafford J.R. 1988. **Evidence for eumelanina and pheomelanin producing genotypes in the arabian horses.** J. Heredity V 79, Issue 2, 100–106,1988

[8] Roberts RJ. **Melanin-containing cells of teleost fish and their relation to disease**. In: Ribelin WE, Migaki G, editors. The Pathology of Fishes. University of Wisconsin p. 399–428, 1975.

[9] Agius C.**The melano-macrophage centres in fish: a review.** In: Fish Immunology (ed. by M.J. Manning & M.F. Tatner), pp. 85–105. Academic Press, London, 1985

[10] Ferguson HW. **The relationship between ellipsoids and melano-macrophage centres in the spleen of turbot (Scophthalmus maximus).** J Comp Pathol 86:377–80. 1976

[11] Ellis AE. **Antigen-trapping in the spleen and kidney of the plaice Pleuronectes platessa** L. J Fish Dis 3:413–26, 1980

[12] Herráez MP, Zapata AG. **Structure and function of the melano-macrophage centres of the goldfish Carassius auratus**. Vet Immunol Immunopathol 12:117–26. doi:10.1016/0165-2427(86)90116-9,1986.

[13] Roberts R.J. **Fish Pathology**, 3rd edn. W.B. Saunders, Philadelphia, PA.,2001

[14] Supamattaya, K.; Fischer-Scherl, T.; Hoffmann, R.W.; BoonyaraTpalin, S**. Light and electron microscopic observations on presporogenic and sporogenic stages of Sphaerospora epinepheli (Myxosporea) in grouper (Epinephellus malabaricus**). Journal of Eukaryotic Microbiology, v. 40, n. 1, p. 71-80, 1993

[15] Silva,A.P.; Silva ,G.R; Cogliati,B;Dias,A.S.M.; Le Bas,A.E. ,Hernandes-Blazquez, F.J. **Bleaching of melnin in the epidermis of South American fur seal and its application on enzyme immunohistochemistry**. Pesq. Vet.Bras. v 31, n 3, 267–270 2011.

[16] Agius C, Roberts RJ. **Melano-macrophage centres and their role in fish pathology**. J Fish Dis 26:499–509, 2003.

[17] Korytowski W. & Sarna T.. **Bleaching melanin pigments: A role of copper ions and hydrogen peroxide in autooxidation and photo-oxidation of synthetic dopa-melanin**. J. Biol. Chem. 265(21):12410-12416, 1990.