

RESEARCH ARTICLE

Occurrence of hepatocellular carcinoma in wild pejerrey *Odontesthes bonariensis* (Teleostei, Atherinidae)

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In non-infectious diseases in fish, neoplasms are of great interest to pathologists because of their similarity to those found in other vertebrates, some of which are generated by similar carcinogens, such as hepatocellular carcinoma (HCC), induced by chlorinated compounds, algae toxins, and mycotoxins. We have studied 100 specimens of *Odontesthes bonariensis* coming from a highly eutrophic lake, San Roque Lake, in Argentina. Hepatomegaly was macroscopically observed in 24 specimens, with external irregular nodular lesions, necrotic, and hemorrhagic areas. The neoplasms were classified according to the WHO for human HCC, in which 20 neoplasms presented a histopathological pattern of the conventional HCC type. Among the neoplasms, four presented a histopathological pattern of sarcomatoid HCC. In the immunohistochemistry, both Hep Par 1, CD34, CD133, and S100 antibodies were positive in all types of HCC and the transmission electron microscopy analysis revealed an ultrastructure similar to that described in the literature for mammalian and human HCC. The etiology of these HCC could not be confirmed, but taking into account the chronic presence of cyanobacteria with frequent algal blooms, we suggest that they are responsible for these neoplasms, especially considering the hepatic carcinogenic activity of algae peptides such as microcystin.

Introduction

Non-infectious pathologies in fish are of great interest to pathologists, and some diseases are very similar and present an evolution and biological behavior similar to mammals and humans, so that today we have an important amount of experimental models of human diseases in fish (Masahito, Ishikawa, and Sugano 1988). Among these diseases, neoplasms are particularly intriguing because of their similarity to those found in vertebrates, thus marking an interesting phylogenetic relationship. There are bioinformatic models that analyze the phylogeny of neoplasms in vertebrates (Schwartz and Schäffer 2017). A large number of neoplasms diagnosed in humans have a counterpart in fish (Mawdesley-Thomas 1971). Some are generated by similar carcinogens, such as HCC, induced by chlorinated compounds, algae toxins (microcystin), and mycotoxins (aflatoxin) (Malins and Ostrander 1994; Pereira 2001; Labine et al. 2015; Saha Turna and Wu 2019). In humans, primary liver cancer is the second leading cause of cancer-related death worldwide and therefore a major public health challenge (Global Burden of Disease Cancer Collaboration 2018).

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The specimens of *O. bonaerensis* studied here came from a highly eutrophic lake, where, along decades, frequent blue-green algae blooms (*Anabaena* and *Microcystis*) were registered and their relative amount is relatively high (Herrero, Sosnovsky, and Morbillo 1998; Morbillo, Sosnovsky, and Herrero 1998). More recently, intense proliferation of the dinoflagellate *Ceratium* have occurred (Marozzi 2019). In the 90's, various histopathological lesions have been reported in various species of fish, including *O. bonaerensis* that inhabit Lake San Roque (Romano et al. 1998).

Cyanobacteria blooming events that produce natural toxins occur in fresh waters around the world, although the potential risk of many cyanobacteria metabolites remains unknown. Only microcystins, a class of cyanopeptides, have been studied intensively, and the large amount of evidence on exposure concentrations and toxicity has led to their inclusion in the water quality risk management model (Janssen 2019).

Materials and methods

We have examined 100 specimens of pejerrey *Odontesthes bonariensis* from Lake San Roque (31°23'S, 64°28'W), Cordoba, Argentina, captured in seven days. The average length of the animals was 21.7 +/- 1.11 cm and the average weight was 217.3 +/- 1.2 g. All specimens were euthanised in an immersion bath with MS-222 of 100 ppm (Western Chemical, USA). The necropsies were performed following a previously described protocol (Romano, Sardella, and Russomando 1987). Fragments of the neoplastic and macroscopically normal liver lesions were fixed in 10% buffered formaldehyde, included in paraplast, sectioned at 3 µm, and stained with hematoxylin and eosin and with Masson's trichromic.

For the immunohistochemistry, the histological sections were stained with an Avidin Biotin Peroxidase complex modified technique (Hsu, Raine, and Fanger 1981). After dewaxing and hydration, the sections were washed in water for 5 min, exposed to a 1% hydrogen peroxide solution in methanol for 30 min to block endogenous peroxidase activity, and washed in phosphate buffered saline (PBS), pH 7.2, for 20 min. After blocking, they were incubated with 3% bovine serum albumin in PBS (BSA, type V Sigma Chemical, St. Louis, Mo.USA) for 40 min. The sections were incubated for 60 min with a primary anti-hepatocyte specific antigen antibody (Hep Par 1) (Roche, Argentina), for 45 min with CD34 antibody (Roche, Argentina), for 40 min with CD133 antibody (Roche, Argentina), and for 90 min with S100 antibody, at a dilution of 1:2500, 1:2000, 1:1500, and 1:1100 in PBS, respectively. Subsequently they were washed in PBS and exposed for 45 min to the biotinyl avidine peroxidase complex (Vectastain ABC kit, Vector). The sections were exposed for 7 min to a 0.1% solution of diaminobenzidine (Polyscience, Warrington, Pa., USA) which was added before use, 0.2% oxygenated water in 50 mM tris buffer, pH 8. Fragments of a human HCC, belonging to the Pathology Department of the



Figure 1.

Longitudinal section of two livers: on the right, an extensive capsulated neoplastic lesion with necrotic-hemorrhagic areas (arrow); to the left, liver with lesion similar to the previous one, also capsulated, with extensive necrotic-hemorrhagic areas (arrow). In the insert a whole liver with two tumor lesions (arrows) Bar: 0.8 cm is observed.

Piñero Hospital in Buenos Aires, Argentina, were used as a positive control. As a negative control in all cases the primary antibodies were replaced by normal rabbit serum at a similar dilution according to Mills (1992).

Tissue fragments of all HCC were fixed for transmission electron microscopy (TEM) in 0.2% glutaraldehyde and post-fixed in osmium tetroxide, then prepared according to the protocol described in previous work (Luchini, Wicki, and Romano 2015). Thick sections stained with toluidine blue were used to locate tumor areas. Ultrafine sections were then prepared and stained with uranyl acetate and lead citrate and examined under a Jeol JEM-8T electron microscope (Jeol, Tokyo, Japan).

Results

In the necropsies, hepatomegaly was found in 24 specimens with external irregular nodular lesions. On cutting, nodular lesions with necrotic and hemorrhagic areas were observed ([Figure 1](#)).

In the sections belonging to the human HCC (positive control), all the antibodies used were marked in the same way as in the fish HCCs. In the sections where the primary antibodies were replaced by normal rabbit serum (negative control), no staining was observed. The HCC neoplasms were classified according to the WHO's criteria for human HCC (Bosman et al. 2010; El Jabbour, Lagana, and Lee 2019). According to WHO criteria 20 neoplasms presented a histopathological pattern typical of the conventional

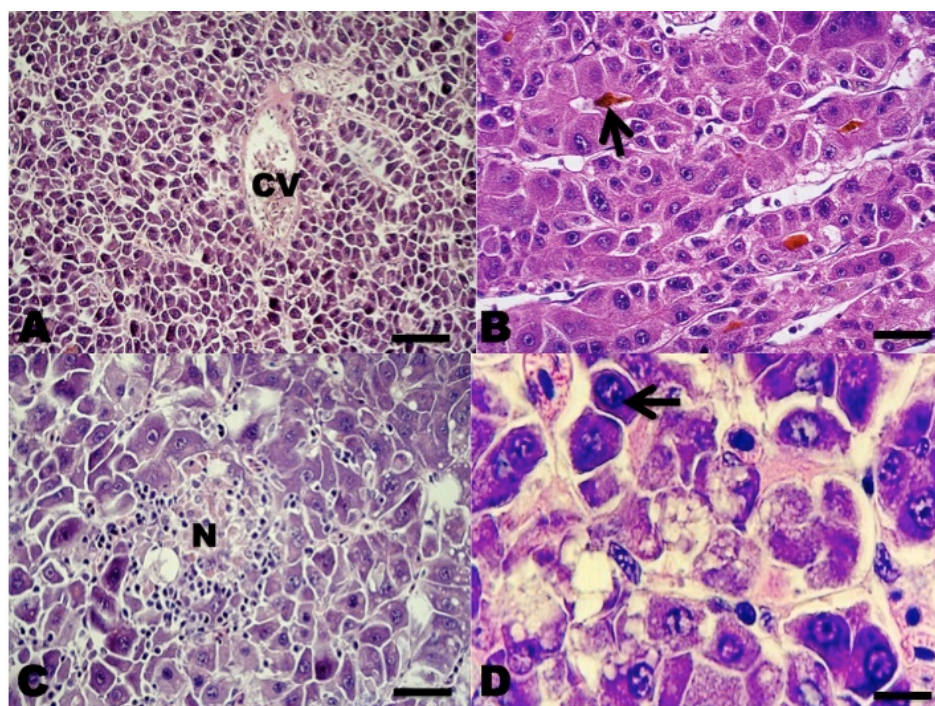


Figure 2.

Conventional HCC. A: Liver parenchyma with its altered architecture, hepatocyte cords arranged in an irregular way; a centrolobulillar vein (CV) is observed. H-E Bar: 200 μ m. B: Irregular cords of atypical hepatocytes with anisocytosis and anisokaryosis. Cholestasis (arrow) may be observed. H-E. Bar: 100 μ m. C: Hepatocytes arranged in a disordered manner with marked anisocytosis and foci of necrosis (N). H-E. Bar: 100 μ m. D: Hepatocytes with marked anaplasia, anisocytosis, anisokaryosis, and nuclear hyperchromatism (arrow). H-E. Bar: 20 μ m.

HCC type, in which a proliferation of liver cells similar to the adult hepatocytes was observed, with little anaplasia, few mitoses, but with changes in the normal liver tissue architecture.

Among the neoplasms, four presented a histopathological pattern of sarcomatoid HCC, in which a proliferation of spindle-shaped cells was observed; few with anaplasia and isolated mitoses with little resemblance to normal hepatocytes, and nests of spindle-shaped cells with fibrosis separating neoplastic lobes (Figures 2 and 3).

In the immunohistochemistry, both Hep Par 1, CD34, CD133, and S100 antibodies were positive in all types of HCC (Figure 4).

In TEM (Figure 5), the neoplastic cells of conventional HCC resembling the normal liver cells. Abundant endoplasmic reticulum and mitochondria were observed. These cells showed aggregates of glycogen in the cytoplasm. Occasionally, clear cells with abundant lipid vacuoles were observed. In the sarcomatoid HCC, spindle cells with numerous osmophilic granules were observed. In addition, mucin-like vacuoles were observed. Isolated narrow cell junctions and microvillus-like prolongations were observed.

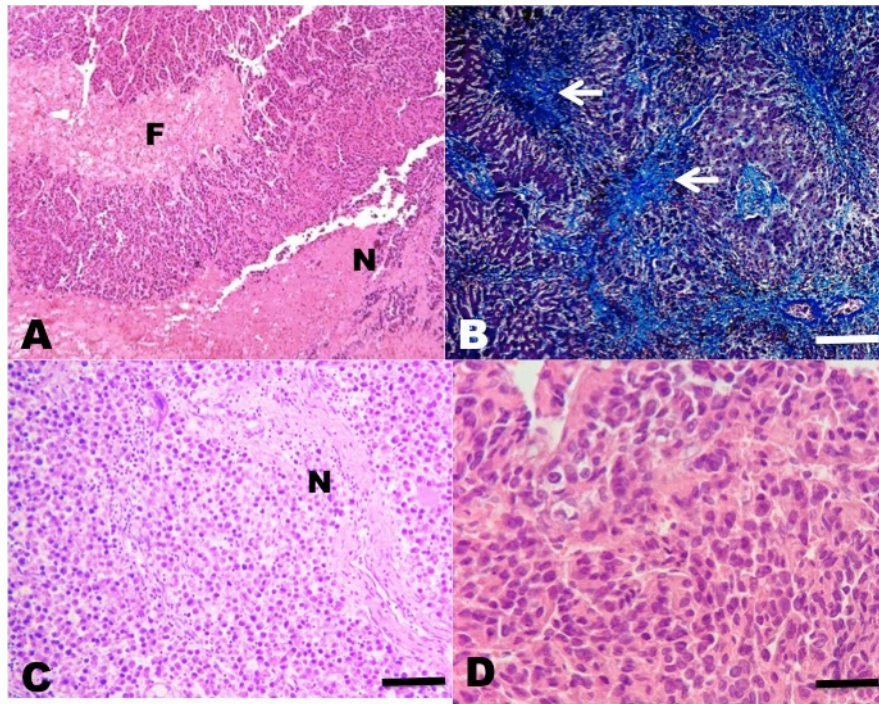


Figure 3.

Sarcomatoid HCC. A: Liver parenchyma with its altered architecture, with fibrosis between hepatocyte cords (F) and necrotic areas (N). H-E. Bar: 200 μ m. B: Liver parenchyma with fibrosis between the atypical hepatocyte cords (arrows). Masson's trichromic. Bar: 200 μ m. C: Cords of atypical hepatocytes with large areas of necrosis (N). H-E. Bar: 200 μ m. D: Sarcomatoid HCC with spindle-shaped cells arranged irregularly with anisocytosis, anikaryosis and mitosis. H-E. Bar: 20 μ m.

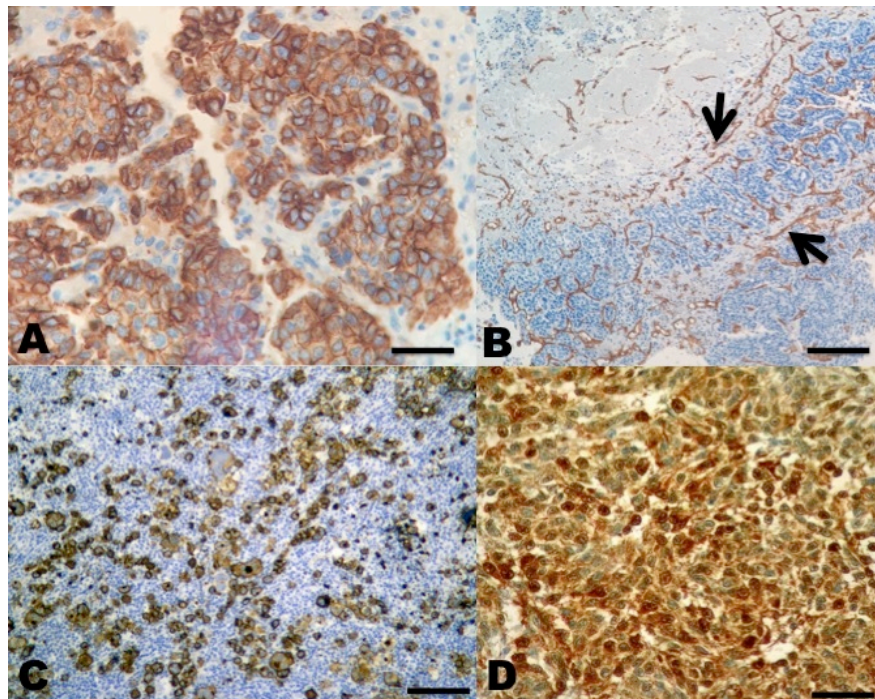


Figure 4.

Immunohistochemistry. A: Conventional HCC with anti-Hep-Par hepatocyte cords. Bar: 50 μ m. B: Sarcomatoid HCC with anti-CD34 positive. Bar: 200 μ m. C: Conventional HCC with anti-C133 positive. Bar: 100 μ m. D: Anti-S100 sarcomatoid HCC positive. Bar: 100 μ m.

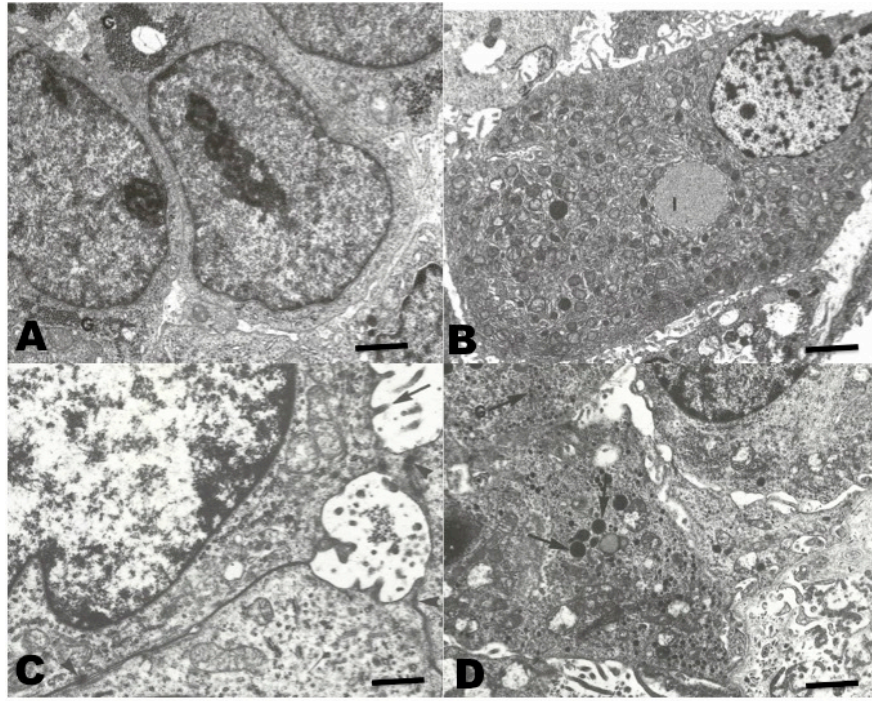


Figure 5.

Electronic microscopy A: Conventional HCC cells rich in glycogen (G), nuclei, and irregular chromatin. Bar: 0.001 μm . B: Neoplastic cells of hepatocellular carcinomas resemble normal liver cells. Abundance of rough endoplasmic reticulum and mitochondria. Presence of an inclusion (I) consisting of interwoven fibers. Bar: 0.001 μm . C: Conventional HCC with narrow cellular junctions (arrowhead) and the main light with microvilli (arrowhead), useful to establish the diagnosis. Bar: 0.001 μm . D: Fibrolamellar HCC, with presence of spindle cells with numerous osmyophilic granules (arrows) and small isolated granules (G arrow). Narrow cellular junctions and microvilli-like extensions are observed. Bar: 0.001 μm .

Discussion

The fourth edition of the World Health Organization (WHO) classification of tumors of the digestive system was published in 2010. In this edition, besides the conventional hepatocarcinoma (HCC), the WHO recognised the following 5 morphological subtypes of HCC: fibrolamellar HCC (FL-HCC), scirrhous HCC (S-HCC), undifferentiated carcinoma, lymphoepithelioma type carcinoma, and sarcomatoid HCC (Bosman et al. 2010; El Jabbour, Lagana, and Lee 2019). The majority of the studied cases (77 %) corresponded to conventional HCC, and 23 % to sarcomatoid HCC. In fish, neoplasms in general are not very frequent, but lately several types of neoplasms have been described in different fish species (Vergneau-Grosset, Nadeau, and Groff 2017). Several cases of HCC have already been described, but due to the low frequency of these neoplasms they have not been classified into histological types, and in the revised bibliographies the HCC subtype has hardly been mentioned. According to Roberts (2012), digestive system neoplasms in general and liver neoplasms are not very frequent. Most articles on HCC are related to experimental models associated with chemicals (Falkner, Emdin, and Ostberg 1976; Aoki and Matsudaira 1977; Wang et al. 2010; Wrighton, Oderberg, and Goessling 2019). Majeed, Jolly, and Gopinath (1984), reported 38 cases of HCC in rainbow trout (*Oncorhynchus mykiss*) from farms, which the authors associated with aflatoxin-contaminated food.

Both well-differentiated hepatocellular neoplastic lesions and poorly differentiated ones represent a diagnostic challenge. “Well-differentiated” implies that the histomorphology of the tumor is very similar to the native tissue from which the tumor originates. Thus, a well-differentiated HCC recapitulates normal liver tissue, mimicking the following benign and preneoplastic entities: hepatocellular adenoma, focal nodular hyperplasia, and regenerative nodule (El Jabbour, Lagana, and Lee 2019). In order to detect malignancy in an apparently normal and well-differentiated hepatic tissue, immunohistochemistry plays a key role in the differential diagnosis, such as application of the monoclonal CD34 antibody, that shows diffuse sinusoidal capillarisation typical of malignant neoplasms (Cui, Wu, and Wen 2018). Both conventional HCC and sarcomatoid HCC showed a histopathological pattern similar to mammals and humans (van Sprundel et al. 2014; Pittman and Brunt 2015; Rastogi 2018).

A panel of positive monoclonal antibodies, also known as anti-Hep-Par1, anti-CD133, anti-CD34, and anti-protein S100 (this was expressed in sarcomatoid type HCC) contributed to the differential diagnosis between benign and malignant lesions, as observed with the anti-hepatocyte specific antigen (Wennerberg, Nalesnik, and Coleman 1993; Torbenson, Y, and Yeh 2018). Lately, immunohistochemistry has represented an important diagnostic tool in the identification of the histogenesis of neoplasias in fish (Romano et al. 2018, 2020; Iaria et al. 2019). Still, immunohistochemistry in these cases is neither 100% specific nor 100% sensitive. Thus, a careful review of histomorphology and the rigorous application of the cytomorphological criteria of malignancy, in addition to the phenotypic expression of the marked antigens, is essential to establish a consistent diagnosis of HCC (Choi, Ramachandran, and Kakar 2017).

CD34 is a glycosylated transmembrane protein that belongs to the salivary mucin family associated with neovascularisation, and is currently considered to be closely related to the development of tumor blood vessels. Therefore, CD34 can be detected in some types of tumors that are associated with blood vessel proliferation (de Boer et al. 2000).

The anti-hepatocyte specific antigen, also known as anti-Hep-Par1, recognises benign and malignant liver derived tissues, including tumors such as HCC. It recognises both normal adult and fetal liver tissue. The typical pattern is a granular cytoplasmic stain. This antibody is useful for differentiating hepatocellular carcinomas with adenoid characteristics from adenocarcinomas, whether primary in the liver or metastatic lesions in the liver (Minervini et al. 1997; Zimmerman et al. 2001; Kuroda and Yorita 2018).

The expression of CD133 is regulated by many extracellular or intracellular factors and represents cellular type changes with particular functions (Wu and Wu 2009). The transforming growth factor $\beta 1$ (TGF $\beta 1$) is able to regulate the increase of CD133 expression specifically within the cellular line of HCC (You, Ding, and Rountree 2010).

During the last decade, it has been discovered that through comparative and functional genomics that several S100 genes are expressed differently in cancer cells. In general, poor tumor differentiation and aggressive neoplasms (Maletzki et al. 2012) have followed the overexpression of S100.

The ultra-structural characteristics of conventional HCC have been widely described for both human and other mammalian HCC (Lapis 1988; Grab, Skubleny, and Kneteman 2019). The cells contain abundant rough endoplasmic reticulum, a relevant number of mitochondria, and glycogen and lipid vacuoles. These ultrastructural characteristics are similar to those found in normal hepatocytes (Baratta et al. 2009). On the other hand, sarcomatoid HCC, also called fibrolamellar, has presented cells which are very different from those of normal hepatocytes; being spindle-shaped, with electrodense granules, and with narrow cell junctions (Andreola, Audisio, and Lombardi 1986; Hasegawa 1996). In all the cases reviewed in this work, they showed an ultrastructure similar to those described in the literature for HCC in mammals and humans.

Regarding the etiology of HCC, in mammals and humans, most cases are related to previous hepatic pathologies, such as cirrhosis and hepatitis B and C, with post-necrotic cirrhosis (Wilson, Mann, and Borthwick 2017; Li et al. 2016; Lin and Kao 2018). Also in mammals, chemical carcinogens are related to the development of HCC (Yang et al. 2019). Non-alcoholic liver metabolic diseases and exposure to diet toxins such as aflatoxins and aristolochic acid are risk factors that can generate HCC (Turner et al. 2005). In fish, in contrast, HCC most often is caused by chemical carcinogens of organic or inorganic origin. Aflatoxins are mycotoxins, which contaminate many basic cereals and oil seeds, being well recognised as liver carcinogens in fish (Anikuttan et al. 2018). Aflatoxin contamination is widespread in areas with a high incidence of HCC. The main form of aflatoxin involved in hepatic carcinogenesis is aflatoxin B1 (AFB1), produced by *Aspergillus* sp. (Wild, Miller, and Groopman 2015).

Cyanobacteria are also considered potential hepatocarcinogens (Svircev et al. 2009) and cyanotoxins represent a very diverse group of toxic substances. They are either attached to the membrane or exist free within the cells. The release of toxins occurs during cell life, but mainly after cell death through passive release of the cell content. Cyanobacterial toxins include neurotoxic alkaloids (anatoxin-a, anatoxin a(s), saxitoxins), hepatotoxic peptides (microcystins), and the hepatotoxic alkaloid (cylindrospermopsin) (Fitzgerald 2001).

Hepatotoxic cyclic peptides include nodularine and different forms of microcystins. Microcystins are a large group of cyclic peptides with varying amino acids in 7 different positions, representing approximately 60 different forms. The most toxic form is microcystin-LR. The toxicity of microcystins is due to their strong union and inhibition of phosphatase proteins (Hitzfeld, Hoyer, and Dietrich 2000; Chorus 2001). It has been demonstrated that after intraperitoneal microcystin injection, the toxin is rapidly found in the liver (Nishiwaki et al. 1994). The presence of microcystin has also been demonstrated in the ovary and liver of fish (Qiao et al. 2019). In mammals the toxic action of microcystin has been described and possibly, its mode of action does not differ from that in fish (McLellan and Manderville 2017). In this work we described 26 cases of HCC in *O. bonariensis* from 100 specimens caught in a highly eutrophic lake, with a history of continuous cyanobacteria blooms. The HCC observed were classified according to WHO, with 20 being conventional HCC and 4 sarcomatoid HCC. The immunohistochemistry confirmed their malignant character and histogenesis.

In this work, the detailed recognition of the tissue and cellular structure of the neoplasms proved an important tool for diagnosis in addition to immunohistochemistry. Although electron microscopy provided a less-specific methodology for the objectives of the study, the ultrastructural findings were as expected and were useful to support the histopathological diagnosis.

The etiology of these HCC could not be confirmed, but considering the chronic presence of cyanobacteria with frequent algal blooms, we suggest that they are responsible for these neoplasms, especially considering the hepatic carcinogenic activity of algae peptides such as microcystin.

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REFERENCES

- Andreola, Salvatore, Riccardo A. Audisio, and Luciano Lombardi. 1986. "A Light Microscopic and Ultrastructural Study of Two Cases of Fibrolamellar Hepatocellular Carcinoma." *Tumori* 72 (6): 609–16. <https://doi.org/10.1177/030089168607200612>.
- Anikuttan, K.K., K.C. George, N.K. Sanil, and K. Sobhana. 2018. "Hepatic Lesions Associated with Induced a Atoxicosis in the Estuarine Teleost *Etrplus Suratensis* (Bloch, 1790." *Indian Journal of Fisheries* 65 (3): 57–65.
- Aoki, Kazuko, and Hiromichi Matsudaira. 1977. "Induction of Hepatic Tumors in a Teleost (*Oryzias Latipes*) After Treatment With Methylazoxymethanol Acetate: Brief Communication 2." *JNCI* 59 (6): 1747–49. <https://doi.org/10.1093/jnci/59.6.1747>.
- Baratta, Janie L., Anthony Ngo, Bryan Lopez, Natasha Kasabwalla, Kenneth J. Longmuir, and Richard T. Robertson. 2009. "Cellular Organization of Normal Mouse Liver: A Histological, Quantitative Immunocytochemical, and Fine Structural Analysis." *Histochemistry and Cell Biology* 131 (6): 713–26. <https://doi.org/10.1007/s00418-009-0577-1>.
- Boer, W. Bastiaan de, Amanda Segal, Felicity A. Frost, and Gregory F. Sterrett. 2000. "Can CD34 Discriminate between Benign and Malignant Hepatocytic Lesions in Fine-Needle Aspirates and Thin Core Biopsies?" *Cancer* 90 (5): 273–78. [https://doi.org/10.1002/1097-0142\(20001025\)90:5](https://doi.org/10.1002/1097-0142(20001025)90:5).
- Bosman, F., F. Carneiro, Hruban R, and N. Theise. 2010. *WHO Classification of Tumours of the Digestive System*. 4th ed. Lyon: IARC.
- Choi, Won-Tak, Rageshree Ramachandran, and Sanjay Kakar. 2017. "Immunohistochemical Approach for the Diagnosis of a Liver Mass on Small Biopsy Specimens." *Human Pathology* 63 (May): 1–13. <https://doi.org/10.1016/j.humpath.2016.12.025>.
- Chorus, Ingrid. 2001. *Cyanotoxins. Occurrence, Causes, Consequences*. Berlin: Springer Berlin Heidelberg. <https://doi.org/10.1007/978-3-642-59514-1>.
- Cui, D.J., Y. Wu, and D.H. Wen. 2018. "CD34, PCNA and CK19 Expressions in AFP-Hepatocellular Carcinoma." *The European Review of Medical and Pharmacological Science* 22: 5200–5205.
- El Jabbour, Tony, Stephen M Lagana, and Hwajeong Lee. 2019. "Update on Hepatocellular Carcinoma: Pathologists' Review." *World Journal of Gastroenterology* 25 (14): 1653–65. <https://doi.org/10.3748/wjg.v25.i14.1653>.
- Falkner, S., S. Emdin, and Y. Ostberg. 1976. "Tumour Pathology of the Hagfish (*Myxine Glutinos*) and the River Lamprey (*Lampr*etra Fluviatilis). A Light Microscopy Study with Special Reference to Primary Liver Carcinoma, Islet Cell Tumors, and Epider- Moid Cysts of the Skin." *Progress in Experimental Tumor Research* 20: 217–50.
- Fitzgerald, J.D. 2001. "Cyanotoxins and Human Health - Overview." In *Cyanotoxins - Occurrence, Causes, Consequences*, edited by I. Chorus, 179–90. Berlin: Springer-Verlag.
- Global Burden of Disease Cancer Collaboration. 2018. "Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived with Disability, and Disability-Adjusted Life-Years for 29 Cancer Groups, 1990 to 2016: A Systematic Analysis for the Global Burden of Disease Study." *JAMA Oncology* 4 (11): 1553. <https://doi.org/10.1001/jamaoncol.2018.2706>.
- Grab, J.G., D. Skubleny, and N.M. Kneteman. 2019. "Cytopathology and Ultrastructure Identification of Primary Hepatic Acinar Cell Carcinoma: Case Report." *International Journal of Surgery Case Reports* 62: 120–25. <https://doi.org/10.1016/j.ijscr.2019.08.006>.
- Hasegawa, Akio. 1996. "Fibrolamellar Hepatocellular Carcinoma: A Case Report of a Resected with an Electron Microscopic and Flow Cytometric Analysis." *Pathology International* 46 (1): 84–90. <https://doi.org/10.1111/j.1440-1827.1996.tb03538.x>.

- Herrero, A.C., A Sosnovsky, and V. Morbillo. 1998. "Estudio de Las Características Bioecológicas Del Lago San Roque, Villa Carlos Paz, Prov. de Córdoba. Parte III: Bioindicadores Hematológicos e Inmunológicos de Contaminación En Peces Teleósteos." *Ingeniería Sanitaria y Ambiental* 39: 83–86.
- Hitzfeld, B.C., S.J. Hoger, and D.R. Dietrich. 2000. "Cyanobacterial Toxins: Removal during Drinking Water Treatment and Human Risk Assessment." *Environmental Health Perspectives* 108: 113–22.
- Hsu, S M, L Raine, and H Fanger. 1981. "Use of Avidin-Biotin-Peroxidase Complex (ABC) in Immunoperoxidase Techniques: A Comparison between ABC and Unlabeled Antibody (PAP) Procedures." *Journal of Histochemistry & Cytochemistry* 29 (4): 577–80. <https://doi.org/10.1177/29.4.6166661>.
- Iaria, C., A. Ieni, I. Corti, R. Puleio, C. Brachelente, G. Mazzullo, and G. Lanteri. 2019. "Immunohistochemical Study of Four Fish Tumors." *Journal of Aquatic Animal Health* 31 (1): 97–106. <https://doi.org/10.1002/aah.10058>.
- Janssen, Elisabeth M.-L. 2019. "Cyanobacterial Peptides beyond Microcystins – A Review on Co-Occurrence, Toxicity, and Challenges for Risk Assessment." *Water Research* 151 (March): 488–99. <https://doi.org/10.1016/j.watres.2018.12.048>.
- Kuroda, Naoto, and Kenji Yorita. 2018. "Clinicopathologic Study of 10 Cases of Gastric Adenocarcinoma with Hepatoid or Enteroblastic Differentiation." *Polish Journal of Pathology* 69 (2): 128–35. <https://doi.org/10.5114/pjp.2018.76696>.
- Labine, Meaghan A., Chris Green, Giselle Mak, Lin Xue, Janet Nowatzki, Jane Griffith, and Gerald Y. Minuk. 2015. "The Geographic Distribution of Liver Cancer in Canada Does Not Associate with Cyanobacterial Toxin Exposure." *International Journal of Environmental Research and Public Health* 12 (12): 15143–53. <https://doi.org/10.3390/ijerph121214969>.
- Lapis, K. 1988. "Ultrastructural Aspects of the Human Primary Liver Cancer." *Applied Pathology* 6 (2): 117–27.
- Li, Yong-Wei, F.C. Yang, H.Q. Lu, and J.S. Zhang. 2016. "Hepatocellular Carcinoma and Hepatitis B Surface Protein." *World Journal of Gastroenterology* 22 (6): 1943. <https://doi.org/10.3748/wjg.v22.i6.1943>.
- Lin, C.-L., and J.-H. Kao. 2018. "Review Article: The Prevention of Hepatitis B-Related Hepatocellular Carcinoma." *Alimentary Pharmacology & Therapeutics* 48 (1): 5–14. <https://doi.org/10.1111/apt.14683>.
- Luchini, Laura, Gustavo Wicki, and Luis Alberto Romano. 2015. "The Ultrastructure of Secretory Cells of the Islets of Langerhans in South American Catfish *Rhamdia Quelen*." *Journal of Histology* 2015 (January): 1–6. <https://doi.org/10.1155/2015/686571>.
- Majeed, S. K., D. W. Jolly, and C. Gopinath. 1984. "An Outbreak of Liver Cell Carcinoma in Rainbow Trout, *Salmo Gairdneri* Richardson, in the U.K." *Journal of Fish Diseases* 7 (2): 165–68. <https://doi.org/10.1111/j.1365-2761.1984.tb00919.x>.
- Maletzki, Claudia, Peggy Bodammer, Anne Breitrück, and Claus Kerkhoff. 2012. "S100 Proteins as Diagnostic and Prognostic Markers in Colorectal and Hepatocellular Carcinoma." *Hepatitis Monthly* 12 (10HCC). <https://doi.org/10.5812/hepatmon.7240>.
- Malins, D.C., and G. Ostrander. 1994. *Aquatic Toxicology Molecular Biochemical and Cellular Perspective*.
- Marozzi, V. 2019. "Análisis de La Floración Algal Recurrente En El Lago San Roque, Córdoba, Argentina." *Ingeniería Sanitaria y Ambiental* 136: 69–78.

- Masahito, Prince, Takatoshi Ishikawa, and Haruo Sugano. 1988. "Fish Tumors and Their Importance in Cancer Research." *Japanese Journal of Cancer Research* 79 (5): 545–55. <https://doi.org/10.1111/j.1349-7006.1988.tb00021.x>.
- Mawdesley-Thomas, Lionel E. 1971. "Neoplasia in Fish: A Review." *Current Topics in Comparative Pathobiology* 1: 87–170. <https://doi.org/10.1016/b978-0-12-153401-1.50008-9>.
- McLellan, Nicole L., and Richard A. Manderville. 2017. "Toxic Mechanisms of Microcystins in Mammals." *Toxicology Research* 6 (4): 391–405. <https://doi.org/10.1039/c7tx00043j>.
- Mills, B. 1992. *Immunohistochemistry*. In *Laboratory Methods in Histotechnology*. Edited by E.B. Prophet, B. Mills, Arrington JB, and L.H. Sobin. Washington, D.C: American Registry of Pathology.
- Minervini, M.I., A.J. Demetris, R.G. Lee, B.I. Carr, Madariaga J, and M.A. Nalesnik. 1997. "Utilization of Hepatocyte-Specific Antibody in the Immunocytochemical Evaluation of Liver Tumors." *Modern Pathology* 10: 686–92.
- Morbillo, V., A Sosnovsky, and A.C. Herrero. 1998. "Estudio de Las Características Bioecológicas Del Lago San Roque, Villa Carlos Paz, Prov. de Córdoba. Parte I: Características Fisicoquímicas y Microbiológicas de Agua y Sedimento." *Ingeniería Sanitaria y Ambiental* 37: 31–36.
- Nishiwaki, Rie, Tetsuya Ohta, Eisaburo Sueoka, Masami Suganuma, Ken-ichi Harada, Mariyo F. Watanabe, and Hirota Fujiki. 1994. "Two Significant Aspects of Microcystin-LR: Specific Binding and Liver Specificity." *Cancer Letters* 83 (1–2): 283–89. [https://doi.org/10.1016/0304-3835\(94\)90331-x](https://doi.org/10.1016/0304-3835(94)90331-x).
- Pereira, M. A. 2001. "Effect of Chloroform on Dichloroacetic Acid and Trichloroacetic Acid-Induced Hypomethylation and Expression of the c-Myc Gene and on Their Promotion of Liver and Kidney Tumors in Mice." *Carcinogenesis* 22 (9): 1511–19. <https://doi.org/10.1093/carcin/22.9.1511>.
- Pittman, Meredith E., and Elizabeth M. Brunt. 2015. "Anatomic Pathology of Hepatocellular Carcinoma: Histopathology Using Classic and New Diagnostic Tools." *Clinics in Liver Disease* 19 (2): 239–59. <https://doi.org/10.1016/j.cld.2015.01.003>.
- Qiao, Qin, Chakib Djediat, Hélène Huet, Charlotte Duval, Séverine Le Manach, Cécile Bernard, Marc Edery, and Benjamin Marie. 2019. "Subcellular Localization of Microcystin in the Liver and the Gonads of Medaka Fish Acutely Exposed to Microcystin-LR." *Toxicon* 159 (March): 14–21. <https://doi.org/10.1016/j.toxicon.2018.12.006>.
- Rastogi, Archana. 2018. "Changing Role of Histopathology in the Diagnosis and Management of Hepatocellular Carcinoma." *World Journal of Gastroenterology* 24 (35): 4000–4013. <https://doi.org/10.3748/wjg.v24.i35.4000>.
- Roberts, R.J. 2012. "Neoplasia of Teleosts." In *Fish Pathology*, 4th ed., 167–85. London: Wiley-Blackwell. <https://doi.org/10.1002/9781118222942.ch5>.
- Romano, Luis Alberto, M.C. Klosterhoff, AF Medeiros, and V.F. Pedrosa. 2020. "Occurrence of Spontaneous Melanoma in Swordtail Fish (*Xiphophorus hellerii*). Study with Optical Microscopy, Immunohistochemistry and Electron Microscopy." *Bulletin of the European Association of Fish Pathologists* 40: 161–67.
- Romano, Luis Alberto, N. Sardella, and F. Russomando. 1987. "La Necropsia En Los Peces. Un Protocolo Tipo Mallory." *Revista de Investigacion y Desarrollo Pesquero* 8: 83–86.
- Romano, Luis Alberto, A. Sosnovsky, A.C. Herrero, and V. Morbillo. 1998. "Estudio de Las Características Bioecológicas Del Lago San Roque, Villa Carlos Paz, Prov. de Córdoba. Parte II: Lesiones Histológicas En Peces Teleósteos Atribuibles a Contaminación Orgánica." *Ingeniería Sanitaria y Ambiental* 38: 62–65.

- Romano, Luis Alberto, G. Wicki, P. Candarte, O.M. Galli, F. Sal, L. Luchini, M Klosterhoff, and V. Pedrosa. 2018. "Melanoma in a *Carassius Auratus* (L.): Study with Optical Microscopy, Electron Microscopy and Immunohistochemistry." *Bulletin of the European Association of Fish Pathologists* 38: 249–57.
- Saha Turna, Nikita, and Felicia Wu. 2019. "Risk Assessment of Aflatoxin-Related Liver Cancer in Bangladesh." *Food Additives and Contaminants Part A: Chemical and Analytical Control Exposure Risk Assessment* 36 (2): 320–26. <https://doi.org/10.1080/19440049.2019.1567941>.
- Schwartz, Russell, and Alejandro A. Schäffer. 2017. "The Evolution of Tumour Phylogenetics: Principles and Practice." *Nature Reviews in Genetics* 18 (4): 213–29. <https://doi.org/10.1038/nrg.2016.170>.
- Sprundel, Renee G.H.M. van, Ted S.G.A.M. van den Ingh, Franco Guscetti, Olivia Kershaw, Monique E. van Wolferen, Jan Rothuizen, and Bart Spee. 2014. "Classification of Primary Hepatic Tumours in the Cat." *The Veterinary Journal* 202 (2): 255–66. <https://doi.org/10.1016/j.tvjl.2014.07.002>.
- Svircev, Z., S. Krstic, M. Miladinov-Mikov, V Baltic, and M Vidovic. 2009. "Freshwater Cyanobacterial Blooms and Primary Liver Cancer Epidemiological Studies in Serbia." *Journal of Environmental Science and Health, Part C* 27 (1): 36–55. <https://doi.org/10.1080/10590500802668016>.
- Torbenson, M., Zen Y, and M.M. Yeh. 2018. *Tumors of the Liver, AFIP Atlas of Tumor Pathology Series 4*. Washington (DC: American Registry of Pathology).
- Turner, PC, A Sylla, YY Gong, MS Diallo, AE Sutcliffe, AJ Hall, and C.P. Wild. 2005. "Reduction in Exposure to Carcinogenic Aflatoxins by Postharvest Intervention Measures in West Africa: A Community-Based Intervention Study." *The Lancet* 365 (9475): 1950–56. [https://doi.org/10.1016/s0140-6736\(05\)66661-5](https://doi.org/10.1016/s0140-6736(05)66661-5).
- Vergneau-Grosset, C, M.-E. Nadeau, and J.M. Groff. 2017. "Fish Oncology: Diseases, Diagnostics, and Therapeutics." *Veterinary Clinics: Exotic Animal Practice* 20 (1): 21–56.
- Wang, Lu, Alvin C. Camus, Wu Dong, Cammi Thornton, and Kristine L. Willett. 2010. "Expression of CYP1C1 and CYP1A in *Fundulus Heteroclitus* during PAH-Induced Carcinogenesis." *Aquatic Toxicology* 99 (4): 439–47. <https://doi.org/10.1016/j.aquatox.2010.06.002>.
- Wennerberg, A.E., M.A. Nalesnik, and W.B. Coleman. 1993. "Hepatocyte Paraffin 1: A Monoclonal Antibody That Reacts with Hepatocytes and Can Be Used for Differential Diagnosis of Hepatic Tumors." *American Journal of Pathology* 143: 1050–54.
- Wild, C.P., J.D. Miller, and J.D. Groopman. 2015. "Mycotoxin Control in Low- and Middle-Income Countries." *IARC* 9.
- Wilson, Caroline L., Derek A. Mann, and Lee A. Borthwick. 2017. "Epigenetic Reprogramming in Liver Fibrosis and Cancer." *Advanced Drug Delivery Reviews* 121 (November): 124–32. <https://doi.org/10.1016/j.addr.2017.10.011>.
- Wrighton, Paul J., Isaac M. Oderberg, and Wolfram Goessling. 2019. "There Is Something Fishy About Liver Cancer: Zebrafish Models of Hepatocellular Carcinoma." *Cellular and Molecular Gastroenterology and Hepatology* 8 (3): 347–63. <https://doi.org/10.1016/j.jcmgh.2019.05.002>.
- Wu, Yaojiong, and Philip Yuguang Wu. 2009. "CD133 as a Marker for Cancer Stem Cells: Progresses and Concerns." *Stem Cells and Development* 18 (8): 1127–34. <https://doi.org/10.1089/scd.2008.0338>.
- Yang, Ju Dong, Pierre Hainaut, Gregory J. Gores, Amina Amadou, Amelie Plymoth, and Lewis R. Roberts. 2019. "A Global View of Hepatocellular Carcinoma: Trends, Risk, Prevention and Management." *Nature Reviews Gastroenterology & Hepatology* 16 (10): 589–604. <https://doi.org/10.1038/s41575-019-0186-y>.

- You, Hanning, Wei Ding, and C. Bart Rountree. 2010. "Epigenetic Regulation of Cancer Stem Cell Marker CD133 by Transforming Growth Factor- β ." *Hepatology* 51 (5): 1635–44. <https://doi.org/10.1002/hep.23544>.
- Zimmerman, Robert L., Melissa A. Burke, Nancy A. Young, Charalambos C. Solomides, and Marluce Bibbo. 2001. "Diagnostic Value of Hepatocyte Paraffin 1 Antibody to Discriminate Hepatocellular Carcinoma from Metastatic Carcinoma in Fine-Needle Aspiration Biopsies of the Liver." *Cancer* 93 (4): 288–91. <https://doi.org/10.1002/cncr.9043>.