International Journal of Advanced Engineering Research and Science (IJAERS)
Peer-Reviewed Journal
ISSN: 2349-6495(P) | 2456-1908(O)
Vol-9, Issue-6; Jun, 2022
Journal Home Page Available: https://ijaers.com/
Article DOI: https://dx.doi.org/10.22161/ijaers.96.45

Preclinical Toxicological Evaluation of the Consumption of Fish from the Cachoeira River Hydrographic Basin in Rats

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Received: 27 May 2022,
Received in revised form: 17 Jun 2022,
Accepted: 22 Jun 2022,
Available online: 30 Jun 2022

Keywords *— **diet, food and nutrition, sustainable development, environmental monitoring, fish, food safety, toxicology.***

Abstract— *Fish is one of the healthiest food sources once it has proteins, vitamins, minerals and the omega-3 polyunsaturated lipids. Nevertheless, to be healthy, the protein in fish meat must not have contaminants further than allowed. This study aimed to investigate the preclinical toxicologic effects of consumption of fish meat from Cachoeira river (Joinville, Santa Catarina, Brazil). Groups of rats were divided and received for a month: standard ration, farmed fish meat and fish meat from Cachoeira river twice a week. One day after the last exposition, animals were euthanized and blood, spleen, heart, liver, kidney, cerebellum, and cerebral cortex were collected to measure oxidative stress, biochemical and hematological parameters. Metals levels were also analyzed in fish meat by atomic emission spectrometry. Significant elevation of carbonylated proteins were observed in heart and liver and thiobarbituric acid reactive substances in liver, plasma and cerebellum were observed. Total*

sulfhydryl content decreased significantly in cerebellum, liver and heart, and decreased catalase activity in the liver and superoxide dismutase activity in the kidneys were also present among rats who consumed fish meat from Cachoeira river. No modification of hematological parameters was observed, and only significant decrease of HDL occurred among biochemical parameters. Analysis of metals in river fish meat showed a fivefold increase in zinc and aluminum compared to farmed fish meat. Short-term exposure to fish meat from the Cachoeira River resulted in increased oxidative stress, liable to be transferred through the food chain, possibly associated with the increased presence of heavy metals.

I. INTRODUCTION

Food is a basic need to ensure survival and to improve the quality of life of the human being, as long as adequate nutrients are consumed for the maintenance of health. The nutrients are divided according to their function in the body into macro and micronutrients and the quantity consumed is defined according to the energy needs of each person and in each phase of life (Ribeiro et al., 2017).

Proteins can be divided into vegetable and animal (from meat, eggs, and fish). Proteins from fish meat are extremely important for health and must be part of routine meals, and its consumption is recommended at least twice a week. Furthermore, fish meat provides vitamins A, E, D, B1, B2, B5, B6, B9, in addition to the minerals calcium and phosphorus, sodium, potassium, manganese, copper and cobalt, zinc, iron and iodine, and also contains all essential amino acids in balance (Food and Agriculture Organization – FAO - 2014). Fish meat is also a source of omega 3, a polyunsaturated lipid with direct action in the prevention of cardiovascular diseases, non-transmissible chronic diseases, and takes part in the neurological development of the fetus and in early childhood (Santos et al., 2013).

To ensure that fish meat is offered and consumed within food safety standards, the place where fish grow and reproduce must be free of contaminations, since they can be transferred to the human body after ingestion in varied degrees, depending on the quantity, length of consumption and level of contamination of the food, and can cause serious health problems, such as predisposition to cancer, cardiovascular diseases and neurotoxicity (Silva & Santos, 2016). The determinant for ensuring the integrity of this source of protein depends on where these fish come from and how this water resource is constituted and whether there is eutrophication that will modify its characteristics (Macedo & Sipaúba-Tavares, 2018).

The problem faced in Brazil and in developing countries is related to most of the raw sewage released without any prior treatment in water courses, containing various types of contaminants, which negatively affect the aquatic environment, which reflects directly on people's health, becoming increasingly necessary the adoption of a practice focused on sustainable development aiming to maintain and preserve natural resources (Campos & Kuhn, 2021; Vincze et al., 2015).

Hence, fish coming from a river with contaminated water can cause damage to the health of the consumers in the long term, due to the exposure to contaminants such as: domestic and industrial effluents, chemical substances from pesticides and fungicides used in agriculture, and heavy metals deposited in the water and which can concentrate in the fish muscle: cadmium (Cd^{+2}), lead (Pb+2), chromium (Cr^{+6}) and mercury (Hg^{+2}) (Lima et al., 2015). The hydrographic basin of the Cachoeira river is located in the central region of the city of Joinville, covers 83.12 Km² in area and represents 7.3% of the city area. Its 100 source is located in the Costa e Silva neighborhood, 40 meters above sea level and its mouth is characterized by an estuary under the influence of tides and where areas with mangrove remnants can be found (Ribeiro & Oliveira, 2014).

In view of the perception of the local social reality of consumption of fish meat from the Cachoeira river, which receives effluents from several origins, including chemical industry, the concern with the long-term exposure of the population to possible contaminants received by the river that may be bioaccumulated in the fish meat arose. Hence, the present study aims to investigate the pre-clinical toxicological effects in rats exposed to the consumption of fish meat from the Cachoeira river basin, which may pose health risks to consumers due to the routing of residential and industrial sewage causing pollution of the river, confirmed by the spatial and temporal variation of water quality index (Oliveira et al., 2013).

II. METHODS

Pre-clinical experimental study, carried out at the University of Joinville Region (UNIVILLE), conducted in the sectorial bioterium and in the laboratories of

pharmaceutical practices and instrumental analysis. The experiments were conducted after approval of the research project by the UNIVILLE's Ethics and Research Committee on Animal Use (Opinion 01/2019).

2.1. Experimental Protocol in vivo

Wistar female albino *Rattus norvegicus*, with an average initial weight of 80-100g, 122 from the Bioterium of the Blumenau Regional University Foundation were used (FURB 123 – SC – Brazil). The animals were received at the age of 21 days, housed (4 per cage) and acclimated for 7 days in the sectorial bioterium of the University of the Joinville Region for adaptation. The animal holding rooms were kept on a 12-h light/dark cycle (lights on at 7:00 am and off at 7:00 pm), temperature between 22 ± 2 °C and humidity between 50 ± 5%, with an air exhaust system. The animals had free access to feed and water. The 128 experiments were carried out according to the provisions of Law No. 11,794 (Brasil, 2008), and other regulations applicable to the use of animals in teaching and/or research, especially the Normative Resolutions of the National Council for the Control of Animal Experimentation (Marques et al., 2009) and the recommendations required by "Guide for the Care and Use of Laboratory Animals (Clark et al., 1996)". After acclimatization, the animals were divided into groups that were exposed for one month to fish meat from farmed fish or from the Cachoeira River, as shown in Table 1:

Table 1. Division of the experimental groups for 1 month exposition to standard ration, farmed fish and Cachoeira river fish meat.

Day of the week	Control group	Farmed fish group	Cachoeira river fish group	Animals per group
Monday	Feed	Feed	Feed	8
Tuesday	8h fasting	8h fasting	8h fasting	8
Wednesday	Feed	Fish	Fish	8
Thursday	Feed	Feed	Feed	8
Friday	8h fasting	8h fasting	8h fasting	8
Saturday	Feed	Fish	Fish	8
Sunday	Feed	Feed	Feed	8

Source: the authors (2022).

The animals in the control group received standard feed (Nuvilab®) and the 139 animals in the experimental groups received 2 servings per week of fish meat. Eight hours before the supply of fish meat, the feed was removed from the boxes of all groups, including the control group. The animals were exposed to fish meat for a period of one month. In order to define the serving of fish offered to each rat, we used the World Health Organization (WHO) recommendation (2014) of consumption of 12 kg of fish per year for an adult of 70 kg (a serving of 125 g/70 kg twice a week).

This proportion was converted according to the body mass of each animal, which was weighed fortnightly, resulting in the following calculation for how much fish would be offered to each animal: 125 g / 70 Kg = 0,001785 µg 149 ∴ 0,00178 µg x body mass of each rat (g) = amount of fish meat offered. One day after the end of each exposure period, the animals were euthanized by decapitation for collection of whole blood, and the spleen, heart, liver, right kidney, heart, cerebral cortex, and cerebellum were excised.

2.2. Sample preparation

2.2.1. Blood

Whole blood for the analysis of hematological parameters was obtained from blood of the rats by decapitation and conditioned in tubes with and without ethylenediaminetetraacetic acid tripotassium (EDTA K3). The blood was centrifuged at 1000 x g for 10 minutes to separate plasma and serum, which were kept in a freezer. The freshly collected blood was used for analysis of hematological parameters and preparation of a slide for differential blood cell count.

Red cells were washed 3 times with ice-cold saline solution (0.153 mol/L sodium chloride) and lysates were prepared by adding 1 mL of distilled water to 100 µL of washed and frozen red cells. For determination of antioxidant enzyme activity, the red cells were frozen and thawed 3 times and centrifuged at 13,500 × g for 10 min. The supernatant was diluted to contain approximately 0.5 mg/mL protein.

2.2.2. Tissue preparation

Liver, kidney, heart, spleen, and brain structures (cortex and cerebellum) were removed, decapsulated and kept on ice in saline buffer (154 mM NaCl, 5 mM Tris-HEPES, pH 7.5). The homogenate (15%) (w/v) was prepared in appropriate buffer 175 according to the methodology to be employed, using Potter-Elvehejem homogenizer (5 pulses). The homogenate was centrifuged at ×3,000 g at 4 °C for 15 minutes to remove cellular debris and the supernatant was stored in aliquots and stored at -80 °C for later determination of the activity of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) and oxidative stress: thiobarbituric acid reactive substances

(TBARS), total sulfhydryl (SH) and carbonylated 181 protein (CP) content.

2.3. Measurement of oxidative stress parameters

2.3.1. TBARS

TBARS were determined according to the method described by Ohkawa et al. (1979). The TBARS methodology measures malondialdehyde (MDA), a product of lipoperoxidation, caused mainly by hydroxyl free radicals. Homogenized tissues and plasma were mixed with 20% trichloroacetic acid and 0.8% thiobarbituric acid and heated in a boiling water bath for 60 min. TBARS were determined by absorbance at 535 nm. A calibration curve was be obtained using 1,1,3,3-tetramethoxypropane as the precursor of MDA and each point of the curve was subjected to the same treatment as that of the supernatants. The results are expressed as nmol of MDA per mg of protein.

2.3.2. Total SH content

Total sulfhydryl content was determined according to the method described by Aksenov & Markesbery (2001), which is based on the reduction of dithionitrobenzoic acid (DTNB) by thiols, generating a yellow derivative, thionitrobenzoic acid (TNB), which is measured spectrophotometrically at 412nm. Briefly, 50µL of homogenate was added to 1 mL of buffer (PBS) pH 7.4 containing 1mM EDTA. The reaction was started by adding 30µL of 10.0mM DTNB and incubated for 30 minutes at room temperature in a dark place. The results are expressed as nmol TNB/mg protein.

2.3.3. CP Content

Carbonyl content was measured using the method described by Reznick & Packer (1994), based on the reaction of protein carbonylation with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm. Briefly, 200 µL of homogenate or plasma were added to plastic tubes containing 400 µL of 10 mM dinitrophenylhydrazine (prepared in 2 M HCl). The samples were kept in the dark for 1 h and vortexed every 15 min. Subsequently, 500 µL of trichloroacetic acid 20% will be added to each tube. The mixture was vortexed and centrifuged at 14,000 x g for 3 min and the supernatant obtained was discarded. The sediment was washed with 1 mL ethanol / ethyl acetate (1: 1 v/v), shaken and centrifuged at 14000 x g for 3 min. The supernatant was discarded and the sediment resuspended in 600µL of 6M guanidine (prepared in a 20 mM potassium phosphate solution, pH 2.3), before vortexing and incubation at 60 °C for 15 min. The samples were then centrifuged at 14,000 x g for 3 min and the supernatant used to measure absorbance at 370 nm (UV) in a quartz cuvette. The results were reported as total carbonyl content (nmol / mg protein).

2.3.4. CAT activity

This parameter was measured by the method of Aebi (1984) using a Shimadzu UV-visible spectrophotometer. The method used is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 25 µL of sample and 600 µL of 10 mM potassium phosphate buffer, pH 7.0, containing 20 mM H_2O_2. The absorbance was measured every 10 seconds for 1 minute 40 seconds. One unit is defined as 1µmol of H_2O_2 consumed per minute and the specific activity is calculated as units of CAT / mg protein.

2.3.5. SOD activity

This parameter was determined by the pyrogallol auto-oxidation method as described by Marklund (1985), a highly superoxide (O2•)-dependent process, which is a substrate for SOD. Briefly, 15 µL of each sample were added to 215µL of a mixture containing 50 µM of Tris buffer, 1 µM of EDTA, pH 8.2, and 30 µM of CAT. Thereafter, 20 µL of pyrogallol were added and the absorbance was recorded immediately every 30 seconds for 3 minutes at 420 nm using a Shimadzu UV-visible spectrophotometer. The inhibition of pyrogallol autoxidation occurs in the presence of SOD, whose activity can be indirectly tested spectrophotometrically. A calibration curve was performed with purified SOD as reference, to calculate the activity of SOD present in the samples. One SOD unit is defined as the amount of SOD required to inhibit 50% of the pyrogallol autoxidation and the specific activity is reported as units/mg of SOD protein.

2.3.6. GSH-Px activity

The measurement of this parameter was performed by the Wendel method (1981), using tert-butyl hydroperoxide as substrate. The decomposition of NADPH was monitored in a spectrophotometer at 340 nm for 3 minutes and 30 seconds (Shimadzu UV-visible spectrophotometer). The medium contains 90 µL of the sample and 800 µL of 10 mM potassium phosphate buffer, pH 7.4; 20 µL of 2 mM GSH, 30 µL of 0.15 U/mL GSH reductase, 10 µL of 0.4 mM azide, and 10 µL of 0.1 mM NADPH. The absorbance was measured every 10 seconds for 1 minute and 30 seconds. Then, 50 µL of 0.5 mM tertbutyl hydroperoxide was added and the absorbance read for another 2 minutes. One unit of GSH-Px is defined as 1µmol of NADPH consumed per minute and the specific activity is presented as GSH-Px units / mg of protein.

2.3.7. Protein dosage

The determination of proteins was performed by the Lowry method (1951), using bovine serum albumin as standard.

2.4. Measurement of biochemical parameters

The biochemical parameters glutamic oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT), thyroid-stimulating hormone (TSH), free thyroxine (FT4) and triiodothyronine (T3) and creatinine were measured by an automatic process in the Siemens Advia Centaur Immunassay System (Santa Helena Laboratory in Jaraguá do Sul – SC - Brazil). The measurement of total protein, albumin, uric acid, urea, total cholesterol (TC), HDL cholesterol and triglycerides (TG) were measured by spectrophotometry in the laboratory of Pharmaceutical Practices at UNIVILLE using Labtest kits. Blood levels of VLDL and LDL cholesterol were deduced using the Friedewald equation.

2.5. Measurement of hematological parameters

The hematological parameters were measured by an automatic process of light absorption and electrical impedance reading through automation with the Horiba ABX Pentra 60 analyzer. The parameters of the red series (hemogram) were evaluated: hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), cellular hemoglobin concentration mean (CHCM), erythrocyte count, RDW (Red Cell Distribution Width), indexes related to hemoglobin concentration and microscopic analysis of the blood smear to observe changes in shape and color of the erythrocytes. The leukogram (total number of leukocytes, neutrophils, eosinophils, lymphocytes, monocytes, and basophils) and the total number of platelets were also evaluated.

2.6. Collecting the fish

Tilapia was selected for this study because it is a widely consumed fish among Brazilians, has a great cost-benefit relation for consumers, and its meat is considered a protein of high biological value and firm texture. This fish can also grow in adverse environments, has omnivorous feeding habits, and feeds on all kinds of organic material available in the water, mollusks, seeds, vegetables of any species, crustaceans, among others (Bemvenuti, Fischer, 2010).

The farmed fish selected were Oreochromis Nile tilapia from a monoculture of tilapia in an excavated and breeding farm located on Quiriri Road in the city of Joinville (SC). The place of culture is regularly attended by the team of the fish farming sector of the rural development unit of the Secretary of Agriculture and Environment, which regularly analyzes the culture water and confirmed that the discriminated culture parameters are within the ideal for tilapiculture. The feed offered to the omnivorous fish has 30% crude protein (Nicoluzzi brand).

The fish were captured with nets, stored in thermal boxes, and immediately delivered to the researcher for cleaning and freezing until the moment of preparation. The fish from the Cachoeira River were collected following the methodology of Lima et al. (2015), with nets, and 3 fish of the same species were captured, stored in Styrofoam boxes with ice until arrival at the UNIVILLE toxicology laboratory, where they were measured and weighed. The fish were collected at most every 3 months, a safe period for storage and maintenance of the quality of the meat for consumption. New collections of fish from the Cachoeira river always occurred 15 days before all available frozen servings were consumed, thus maintaining the collection and consumption period within the 3-month period. The collection of fish was performed during the day, always considering the condition of no rainfall for at least two days before the collections and taking into account the ease of access and the presence of the target species (Pinheiro et al., 2015). The tilapia was collected in the same stretch of the Cachoeira River (coordinates 26°16084'S r048°51.880' W), in the Costa e Silva neighborhood, in Joinville (SC). The fish were collected using 4-cm mesh cast nets and 8-meter long 1-meter wide 6-cm mesh gillnets.

After being captured, the individuals were identified to the lowest taxonomic level possible to confirm that it was the target species, placed in plastic bags, weighed, labeled, sealed, and stored in a thermal box with ice. Nineteen individuals were collected, totaling 5100 g of tilapia. The collection procedures were conducted after permission from the Biodiversity Authorization and Information System (SISBIO) No. 10476-3 by the biologists Diogo Augusto Moreira (Regional Council of Biology - CrBio - 81154) and Johnatas Adelir-Alves (Regional Council of Biology-CrBio - 053967).

2.7. Preparation of the fish meat

The selection and preparation of the fish meat for consumption by the rats followed the food safety standards of the Collegiate Directorate Resolution (CDR) No. 216, of September 15, 2004, which provides on the Technical Regulation of Good Practices for Food Services (Agência Nacional de Vigilância Sanitária – ANVISA - 2004). Soon after the fish was caught, it was cleaned, gutted, and its scales and head were removed. The flesh of the farmed fish and of the Cachoeira river was separated from the bones and only the raw, filleted meat was packed individually or a maximum of two fillets in transparent

plastic bags selected according to CDR standard No. 259/2002 (Brasil, 2002), kept at a temperature between 0°C and -2°C until the moment of thawing and cooking of the meat. The thawing of the meat occurred under refrigeration conditions at a temperature below 5 ºC, to prevent the surface areas of this food from remaining in conditions favorable for microbial multiplication.

The cooking was carried out in a glass container, only in drinking water without any added seasoning, at a temperature of 150 °C for 10 minutes (Brasil, 2004). After cooking, the fish meat was cooled, separated into servings and then frozen at -5 °C, packed in plastic bags according to the CDR No. 259/2002 to ensure the nutritional safety of this food and minimize the risk of cross contamination. The meat was thawed in a microwave oven for 2 minutes before being offered to each animal. Next, it was packaged in a thermal bag for transport to the bioterium, where each serving was weighed on precision scales (Brasil, 2018; Cribb et al., 2018). The fish meat was offered to the animals twice a week, after an 8-hour fasting period. Each animal was placed individually in a cage to ensure that each one consumed its serving, and after verification of total consumption of the serving, they were placed back in the larger box together with the other animals in their group and unlimited feed was made available again. Leftovers of the thawed fish meat after consumption by the rats were not reused.

2.8. Heavy metals in fish meat quantification

The analyses of heavy metals concentration in fish meat were conducted in triplicate by inductively coupled plasma atomic emission spectroscopy (ICP-OES) in an Avio 200-Perkin Elmer instrument, adapted from the methodologies of Sanches Filho et al. (2013) and Uysal et al. (2008). 5 g of each sample of farmed fish meat and from the Cachoeira River were weighed and placed in a lyophilizer for 24 hours. After drying, 3.40 g of the farmed fish meat sample and 3.22 g of the fish meat sample from the Cachoeira river were left. To each sample 10 mL HCl (20%) + 4mL HNO3 (50%) were added, and they were placed on the heater plate at 90°C and left for 30 minutes in reflux. The samples were then filtered into a 100 mL flask and volumized with Milli-Q water. All the material used in the sample preparation was washed with a solution of Extran and water and then placed in 10% (v/v) nitric acid for at least 24 hours. After immersion in the acid, the material was washed seven times in Milli-Q water. The results of the metal analysis are expressed in ppm.

2.9. Statistical Analysis

Results are presented as mean ± standard deviation (metal level measurement) or mean ± standard error (oxidative stress, biochemical and hematological parameters), were tabulated and analyzed using GraphPad Prism 6.0 software. Analysis of variance (ANOVA), followed by Kruskal-Wallis post-test (non-parametric data) or Tukey's test (parametric data), for comparison among groups was conducted, with values of $p < 0.05$ considered significant.

III. RESULTS

No statistically significant differences were observed for oxidative stress parameters and antioxidant enzymes in the spleen and cerebral cortex among the groups (Table 2) and there were no relevant differences between animals fed with feed and farmed fish. In the cerebellum a significant elevation of TBARS (24.0%) and a relevant decrease of SH (30.8%) were observed in the animals fed with the fish from the polluted river, and although there was a decrease in GSH-Px activity, no statistical relevance was 379 reached ($p = 0.104$) and no changes in the activity of the other antioxidant enzymes were found. In the heart, there was a significant decrease of SH (15.9%) and a relevant increase of the total content of CP (56.2%), but there were no relevant changes in the activity of antioxidant enzymes and lipid peroxidation in this organ. A significant elevation of TBARS (15.9%) and CP (50.0%) was observed in the liver, as well as a relevant reduction of SH (14.8%) in animals that received fish from the Cachoeira River.

Moreover, the enzymatic activity of SOD suffered statistically significant reduction in the liver (11.0%) of the animals that received the fish meat from the polluted river. In the kidney, the elevation in total CP content did not reach statistical relevance ($p = 0.07$), however it is not insignificant considering that it is only one month of exposure to the food. Among the antioxidant enzymes, a significant reduction (25.5%) in CAT activity was found in this organ in the animals fed with the fish meat from the polluted river. In plasma, a 79.4% elevation of TBARS was found in the group of animals that consumed the fish meat from the polluted river compared to the other groups. There was no significant modification of the other parameters or in the activity of antioxidant enzymes in erythrocytes (Table 3).

Table 2 - Oxidative stress parameters after one month of exposure to standard ration, farmed fish, and fish meat from the Cachoeira river.

Organ (Group)	TBARS (nmol/mg of protein)	SH (nmol/mg of protein)	CP (nmol/mg of protein)
Spleen (Feed)	3.1 ± 0.1	33.2 ± 1.5	7.2 ± 0.1

Spleen (Farmed)	3.1 ± 0.1	34.4 ± 1.3	6.7 ± 0.2
Spleen (Cachoeira)	3.1 ± 0.1	31.1 ± 1.9	7.3 ± 0.2
Cerebellum (Feed)	4.3 ± 0.1	25.1 ± 1.6	5.2 ± 0.1
Cerebellum (Farmed)	4.2 ± 0.2	22.2 ± 1.3	5.1 ± 0.1
Cerebellum (Cachoeira)	**5.42 ± 0.3***	**17.39 ± 1.1***	5.27 ± 0.1
Cortex (Feed)	3.1 ± 0.2	22.2 ± 0.7	5.1 ± 0.1
Cortex (Farmed)	2.8 ± 0.1	22.5 ± 0.6	5.1 ± 0.1
Cortex (Cachoeira)	3.2 ± 0.2	21.8 ± 0.7	5.2 ± 0.1
Heart (Feed)	4.0 ± 0.1	36.3 ± 1.8	5.5 ± 0.1
Heart (Farmed)	4.1 ± 0.1	35.3 ± 1.2	5.26± 0.2
Heart (Cachoeira)	4.2 ± 0.1	**30.5 ± 1.3***	**8.61± 0.2*****
Liver (Feed)	2.5 ± 0.1	89.6 ± 5.2	4.1 ± 0.2
Liver (Farmed)	2.5 ± 0.1	97.4 ± 2.5	4.6 ± 0.1
Liver (Cachoeira)	**2.9 ± 0.1***	**76.2 ± 3.9****	**6.1 ± 0.2*****
Kidney (Feed)	2.5 ± 0.1	31.7 ± 1.4	3.4 ± 0.2
Kidney (Farmed)	2.6 ± 0.1	30.3 ± 1.0	3.3 ± 0.2
Kidney (Cachoeira)	2.6 ± 0.1	31.4 ± 0.6	4.0 ± 0.1
Plasma (Feed)	3.48 ± 0.21	31.04 ± 0.98	Not measured
Plasma (Farmed)	3.43 ± 0.16	29.20 ± 1.06	Not measured
Plasma (Cachoeira)	**6.10 ± 0.26*****	28.62 ± 0.71	Not measured

Source - the authors (2022).

* Statistically significant difference between feed and farmed fish groups ($p < 0.05$).

** Statistically significant difference between feed and farmed fish groups ($p < 0.01$).

*** Statistically significant difference between feed and farmed fish groups ($p < 0.001$).

Table 3 – Antioxidant enzymes activity after one month of exposure to standard ration, farmed fish, and fish meat from the Cachoeira river.

Organ (Group)	CAT (U/mg of protein)	SOD (U/mg of protein)	GSH-Px (U/mg of protein)
Spleen (Feed)	10.2 ± 0.8	9.8 ± 0.2	37.7 ± 1.6
Spleen (Farmed)	9.9 ± 0.6	9.7 ± 0.2	37.5 ± 1.6
Spleen (Cachoeira)	12.0 ± 0.7	9.5 ± 0.1	36.0 ± 1.9
Cerebellum (Feed)	6.8 ± 0.1	5.1 ± 0.1	26.4 ± 0.7
Cerebellum (Farmed)	6.95 ± 0.1	4.72 ± 0.1	29.4 ± 2.0
Cerebellum (Cachoeira)	7.09 ± 0.1	4.69 ± 0.1	25.0 ± 1.8
Cortex (Feed)	7.0 ± 0.1	6.4 ± 0.3	28.8 ± 1.8
Cortex (Farmed)	6.9 ± 0.2	6.4 ± 0.3	28.0 ± 1.3
Cortex (Cachoeira)	7.0 ± 0.1	7.3 ± 0.4	29.6 ± 1.3
Heart (Feed)	9.3 ± 0.5	5.7 ± 0.1	60.3 ± 3.1
Heart (Farmed)	9.1 ± 0.3	5.8 ± 0.1	61.7 ± 2.0
Heart (Cachoeira)	8.4 ± 0.3	5.7 ± 0.1	60.5 ± 1.1
Liver (Feed)	15.2 ± 0.6	6.3 ± 0.2	64.5 ± 2.4
Liver (Farmed)	15.1 ± 0.5	6.0 ± 0.1	59.0 ± 1.5

Liver (Cachoeira)	15.5 ± 0.5	**5.6 ± 0.1***	58.5 ± 3.5
Kidney (Feed)	15.4 ± 0.6	6.3 ± 0.1	32.6 ± 1.3
Kidney (Farmed)	15.6 ± 0.5	6.4 ± 0.1	32.4 ± 1.8
Kidney (Cachoeira)	**11.5 ± 0.5***	6.8 ± 0.1	33.8 ± 1.6
Erythrocytes (Feed)	2.75 ± 0.16	5.93 ± 0.08	25.10 ± 1.29
Erythrocytes (Farmed)	2.70 ± 0.10	6.30 ± 0.12	25.40 ± 1.52
Erythrocytes (Cachoeira)	2.62 ± 0.08	5.98 ± 0.16	23.76 ± 1.27

Source - the authors (2022).

* Statistically significant difference between feed and farmed fish groups ($p < 0.05$).

The thyroid and kidney functions of the animals were not significantly modified in any of the groups (Table 4). The lipidogram showed a significant reduction in HDL cholesterol for the consumption of both farmed fish and fish from the polluted river. There was a tendency of alteration in the hepatic function, verified through the increase of GTP, but it did not reach statistical significance ($p= 0.07$).

Table 4 – Biochemical parameters after one month of exposure to standard ration, farmed fish, and fish meat from the Cachoeira river.

Parameter	Feed (n= 8)	Farmed Fish (n = 8)	Fish from Cachoeira (n = 8)
TSH (µU/mL)	0.012 ± 0.002	0.013 ± 0.002	0.012 ± 0.001
FT4 (ng/dL)	1.442 ± 0.032	1.548 ± 0.007	1.538 ± 0.005
T3 (ng/mL)	0.462 ± 0.011	0.491 ± 0.003	0.500 ± 0.002
GOT (U/L)	245.40 ± 26.76	280.25 ± 28.01	237.00 ± 30.75
GPT (U/L)	67.00 ± 16.04	80.50 ± 15.66	102.66 ± 9.22
Total Proteins (g/dL)	5.91 ± 0.07	5.71 ± 0.19	6.04 ± 0.19
Albumin (g/dL)	2.31 ± 0.19	2.68 ± 0.09	2.64 ± 0.03
Urea (mg/dL)	49.87 ± 4.86	55.84 ± 4.56	53.87 ± 3.22
Creatinine (mg/dL)	0.73 ± 0.03	0.67 ± 0.02	0.68 ± 0.01
Uric acid (mg/dL)	1.62 ± 0.24	2.08 ± 0.26	2.00 ± 0.31
Total Cholesterol (mg/dL)	222.20 ± 12.89	207.80 ± 7.59	183.30 ± 7.01
C-HDL (mg/dL)	55.00 ± 5.99	**31.60 ± 2.11***	**26.83 ± 0.70***
TG (mg/dL)	171.60 ± 7.43	188.80 ± 5.11	189.40 ± 8.75
C-LDL	132.90 ± 15.89	136.00 ± 7.72	115.50 ±7.26
C-VLDL	34.32 ± 1.48	37.77 ± 1.02	37.89 ± 1.75

Source - the authors (2022).

* Statistically significant difference compared to the feed group ($p < 0.05$).

No statistically significant differences were found between the groups for the total erythrocyte, leukocyte, or platelet count, as well as for the differential leukocyte count and other erythrogram parameters (Table 5).

While farmed fish meat samples showed no relevant modification of heavy metals content, fish meat from the Cachoeira River showed an average five-fold significant increase of Al^{+3} and Zn^{+2}. The increase in Cr^{+6} and Cd^{+2} in the meat from the Cachoeira River compared to the farmed fish did not reach statistical significance, but this does not exclude the possibility of bioaccumulation due to chronic exposure over longer periods of exposure. There was no significant difference in the amounts of Ni^{+2}, Pb^{+2}, Cu^{+2} and Hg^{+2} among the samples (Table 6).

Table 5 – Hematologic parameters after one month of exposure to standard ration, farmed fish, and fish meat from the Cachoeira river.

Parameter	Feed (n = 8)	Farmed fish (n = 8)	Fish from Cachoeira (n = 8)
Total leukocytes (x1000/µL)	4.76 ± 0.60	4.26 ± 0.26	4.10 ± 0.76

Segmented (%)	17.80 ± 2.17	22.00 ± 1.67	17.12 ± 1.00
Rod Cells (%)	0	0	0
Lymphocytes (%)	70.60 ± 2.15	66.16 ± 1.49	64.87 ± 1.14
Monocytes (%)	9.60 ± 0.67	9.33 ± 0.61	12.50 ± 1.06
Eosinophils (%)	2.00 ± 0.54	1.83 ± 0.83	3.25 ± 0.36
Basophils (%)	0	1.00 ± 0.25	1.00 ± 0.26
Total erythrocytes (million/mm3)	7.10 ± 0.14	7.03 ± 0.10	6.99 ± 0.09
Hemoglobin (g/dL)	14.84 ± 0.22	14.31 ± 0.43	14.52 ± 0.19
Hematocrit (%)	39.50 ± 0.51	38.61 ± 1.21	38.83 ± 0.48
MCV (fL)	55.80 ± 0.66	55.50 ± 0.61	55.50 ± 0.32
MCH (ng)	20.86 ± 0.44	20.66 ± 0.31	20.80 ± 0.19
CHCM (g/dL)	37.54 ± 0.31	37.15 ± 0.25	37.42 ± 0.20
RDW (%)	10.94 ± 0.25	10.80 ± 0.23	11.07 ± 0.12
Total platelets (x 1000/µL)	634.50 ± 54.43	639.00 ± 102.2	746.9 ± 31.82

Source - the authors (2022).

Table 6 – Quantity of heavy metals in fish meat from farmed fish and from the Cachoeira river

Metal (ppm)	White	Farmed Fish	Fish from Cachoeira
Al^{+3}	16.600 ± 0.008	10.580 ± 0.073	**56.520 ± 0.018***
Cr^{+6}	0	1.170 ± 0.029	4.35 ± 0.007
Zn^{+2}	0.155 ± 0.006	59.700 ± 0.002	**381.900 ± 0.003*****
Ni^{+2}	0	0	0
Pb^{+2}	0	0	0
Cu^{+2}	0.014 ± 0.028	2.940 ± 0.006	3.720 ± 0.019
Cd^{+2}	0	0	0.930 ± 0.056
Hg^{+2}	0	0	0

Source - the authors (2022).

IV. DISCUSSION

In the present study, among the animals that consumed fish meat from the Cachoeira river, a significant increase in lipid peroxidation was observed in the cerebellum, liver, and plasma, and also an increase in CP in the heart and liver. A relevant reduction of SH occurred in the heart and liver. These data highlight the increased oxidative stress to which the animals were exposed by consuming fish meat with significantly higher levels of Al+3 and Zn+2 compared to farmed fish meat. Still, there was a decrease of SOD activity in the liver and CAT activity in the kidneys. The hepatic toxicity is remarkable, which, if considered associated with the elevation of GPT (even though it did not reach statistical significance), is not negligible considering that the exposure period was only one month.

TBARS are biomarkers of lipoperoxidation derived from the reaction of malondialdehyde with thiobarbituric acid (Keshari et al., 2015). SH groups are the largest and most frequent antioxidants in plasma and the erythrocyte membrane contains high concentration of these groups, which can be converted by oxidizing agents into disulfide agents and cause denaturation of membrane proteins, and intracellular damage can also occur, such as oxidation of hemoglobin into metahemoglobin (Van Dijk et al., 2020).

CPs are formed by structural oxidative cleavage of proteins, by deamination of amino acids, such as lysine and glutamic acid, and their increase is present in conditions such as: aging, neurodegenerative diseases, obesity, diabetes, age-related macular degeneration, anemia, hepatocellular carcinoma, acquired immunodeficiency syndrome (Frijhoff et al., 2015). Due to the widespread potential for cellular damage caused by the imbalance between ROS production and antioxidant defense, the protective elements include endogenous enzymatic and non-enzymatic systems. The former includes the enzymes: SOD, which performs the dismutation of superoxide ions by accelerating their conversion to hydrogen peroxide in the mitochondria and cytosol; CAT, which converts hydrogen peroxide to water and oxygen; and GSH-Px, which is the most important H2O2 scavenging enzyme using glutathione as a cofactor, and the non-enzymatic systems include ROS scavengers such as vitamins A, C, and E (Keshari et al., 2015).

Al^{+3} is the third most common element in the earth's crust, has a wide variety of uses, and its concentration in beverages and food has increased because of soil acidification, which allows greater transfer of this metal to the aquatic medium, and anthropogenic activities, such as mining and industry. Diet is the main form of exposure to metals, and Al^{+3} is found in water, where it is added as a flocculant ($Al_2(SO_4)_3$), processed foods, packaging, and in fresh foods (vegetables and fruit) due to its presence in the soil. Also, utensils containing Al^{+3} are responsible for its increased presence in the diet. However, it has no function in the human and animal organism and tends to accumulate in the brain, bones, liver and kidneys, so that chronic exposure can cause relevant toxicity, since Al^{+3} can: interfere with the function of calcium and replace it in bone mineralization and cell growth, as well as increase the stability of iron regulatory protein 2 (cytosolic protein that helps maintain iron level), trigger the development of Alzheimer's disease and other neurological and cognitive disorders (Hardison et al., 2017).

The pathophysiological mechanisms surrounding Al^{+3} toxicity include very basic cellular functions, such as enhancement of oxidative stress damage, which results in oxidation of proteins and lipids by free radicals (Igbokwe et al., 2019) and decreased activity of the antioxidant enzymes CAT, SOD and GSH-Px (Slaninova et al., 2014), in addition to disrupting calcium-mediated intracellular signaling, which will systematically lower cellular defenses, inhibit enzyme action, and impair mitochondrial function, thus making it a great villain of the tissues in which it accumulates, and can cause anything from anemia and hepatic or renal toxicity to neurodegenerative and reproductive diseases (Exley, 2014).

Yuan et al. (2012) found significant accumulation of AlCl3 in newborn rats exposed for 14 days intraperitoneally once a day (doses of 0.7 and 35 mg/Kg of $AlCl_3$) in the cerebellum, hippocampus, and diencephalon of the animals, in addition to significant increase of TBARS and decrease of SOD activity but increase of GSH-Px activity in these regions. The authors explain that brain tissue is more susceptible to oxidative damage because it consumes more oxygen, has more polyunsaturated fatty acids in cell membranes, high iron content, and low activity of antioxidant enzymes. Al^{+3} can cause oxidative damage to the brain by binding to negatively charged phospholipids, which contain polyunsaturated fatty acids that are easy targets for ROS, stimulating iron-induced lipid peroxidation (Fenton reaction), and directly forming Al-O_2^- from the reaction of Al^{+3} with superoxide anion, which elevates the oxidative capacity of O_2.

Labbar et al. (2021) observed significant drop in CAT and SOD activity in astrocyte culture incubated with $AlCl_3$. In rats exposed for only three days to different doses (25, 50 and 100 mg/Kg, intraperitoneally) of $AlCl_3$, there was significant astrogliosis in the motor cortex and hippocampus of the animals.

Nathyia & Nandhini (2014) evidenced the toxicity on the antioxidant system of $AlCl_3$ (300 mg/Kg, orally, once a day, for 31 days) in several organs: they observed significant increase of TBARS in liver, kidney, heart, and lungs of rats, as well as drop of CAT, SOD and GSH-Px activity. Hawas et al. (2020) found that exposure to $AlCl_3$ 100 mg/Kg, orally, for 21 days (once daily) caused edema, disorganization of myofibrils and destruction of cardiac striated muscles. Liu et al. (2016) found in 5-week-old rats that $AlCl_3$ included in the water ingested by the animals, at all doses (0.4; 0.8 and 1.6 g/L) significantly lowered GSH-Px activity, that the higher dose reduced SOD activity, and that doses of 0.8 and 1.6 g/L significantly increased the level of TBARS.

Othman et al. (2017) reaffirmed the preclinical hepatic and renal toxicity in rats (ten weeks old) that received $AlCl_3$ (34 mg/Kg, orally) for eight weeks and resulted in significant increase in TBARS and reduced activity of CAT, SOD and GSH-Px, in addition to increasing the level of inflammatory cytokines in these organs (interleukin 1beta and tumor necrosis factor alpha). Degeneration, vacuolization, and cellular necrosis were found in the hepatocytes and apoptosis and glomeruli collapse in the kidneys, and also increased blood activity of GOT, GPT, and bilirubin concentration (biomarkers of liver function) and urea and creatinine (biomarkers of kidney function). Chary et al. (2017) observed significant drop in blood level of HDL, VLDL, and triglycerides in rats that received aqueous $AlCl_3$ solution (50 and 100 mg/Kg, orally, once daily) for 28 days. Histological toxicity was also observed which may have contributed to lower hepatic synthesis of lipoproteins. Gaballa et al. (2013) also found significant reduction in blood HDL level in humans occupationally exposed in a factory to Al^{+3} dust between 0.33-3.4 mg/m3 in air (which was still within the acceptable limit - 5 mg/m3).

The accumulation of Al^{+3} in hepatocytes may affect protein secretion and vacuolization promoting hepatosteatosis, which is the accumulation of lipids in cytoplasmic vesicles (Belaïd-Nouira et al., 2013), motivating dyslipidemia mediated by Al^{+3} toxicity in hepatocytes. In animals that consumed farmed fish meat, the drop in HDL found may be due to contaminants other than heavy metals.

Zn^{+2} is an essential mineral for humans, since it acts as a cofactor for more than 300 enzymes and 200 transcription factors, besides being an important mediator of cell signaling. In the antioxidant system, Zn^{+2} protects cells against oxidative stress because it acts as a membrane stabilizer, is a cofactor for SOD and GSH-Px, inhibits the pro-oxidant enzyme, nicotinamide adenine dinucleotide phosphate oxidase, and promotes the synthesis of metallothionein, which catalyzes the reduction of hydroxyl radicals and sequesters ROS. Further, Zn^{+2} competes with iron and Cu^{+2} for binding sites on the cell membrane, preventing lipid lipoperoxidation caused by them, since it is catalytically inert (Marreiro et al., 2017). Zn^{+2} also interacts with thiol or SH groups on proteins and peptides, reducing their susceptibility to oxidation (Choi et al., 2018).

Zn^{+2} is neither cytotoxic, carcinogenic, mutagenic, nor teratogenic, and intoxications with this metal are rare and primarily related to Cu^{+2} deficiency. On the other hand, Zn^{+2} deficiency presents a risk for the health of individuals, since, after iron, it is the second most prevalent trace element in the human body, and its lack is related to: growth retardation, drop in immunity, increased oxidative stress and synthesis of inflammatory cytokines, mental lethargy, cognitive impairment, symptoms of depression, and Alzheimer's disease (Jarosz et al., 2017). Still, the protective effect on rat brain oxidative stress mediated by Al^{+3} and Cd^{+2} ($ZnCl_2$ 30 and 60 mg/L in water for 60 days) and also on liver and kidneys of diabetic rats ($ZnSO_4$ mg/Kg for 21 days) for therapeutic exposure to Zn^{+2} was described by Brzóska et al. (2021), corroborating its protective potential. However, at the intracellular level, excess free Zn^{+2} promotes the activation of protein kinase C, which leads to the production of ROS via NADPH oxidase. Mitochondria and lysosomes are Zn^{+2}-storing organelles that, when having Zn^{+2} in excess, suffer membrane permeabilization, and the release of their contents may trigger cell death, as well as the activation of the caspase-3 pathway. Hence, in excess, Zn^{+2} can become cytotoxic (Kim et al., 2020).

Singh et al. (2012) exposed rats to diet with 20, 40 and 80 mg/Kg of Zn+2 for 6 months and observed in blood increased TBARS, increased activity of CAT, SOD and GSH-Px enzymes, increased blood level of triglycerides, LDL, VLDL and decreased HDL for the two highest doses. These data were associated with higher concentration of Zn^{+2} and lower concentration of Cu^{+2}, Mg^{+2} and Mn^{+2} in liver and kidney of the animals, suggesting that excess of chronic Zn^{+2} may result in change of oxidative stress by altering the level of other minerals. Taken together, these studies suggest that Zn^{+2} does not have a solely antioxidant function but may also be pro-oxidant.

Accordingly, the findings of the present study are understood, which were parallel to the toxicity mechanisms in the sites where the accumulation of Al^{+3} was sufficient to evoke increased oxidative stress (cerebellum, heart, liver, and plasma) and decreased activity of antioxidant enzymes (liver and kidney). Nonetheless, it is important to emphasize that in the present study the exposure was related to alterations linked to the consumption of fish meat in nutritionally recommended amounts and that it occurred in the second trophic level of the food chain, suggesting that oxidative stress and relevant toxicity may arise from chronic ingestion of food contaminated with heavy metals.

Reduced synthesis and secretion of erythropoeitin, of heme group and globulins, inhibition of intestinal iron absorption and increased hemolysis are mechanisms that attribute anemia to Al^{+3} (Ige & Aiyeola, 2017). Nevertheless, no hematological parameters were changed in the present study, possibly due to the low exposure time. As a result of acidification of soils, soluble Al^{+3} can reach the aquatic environment easily, hence seafood is the greatest accumulator of this metal, with an average concentration of 204.6 mg/Kg having been found in sea squirts from South Korea (Choi et al., 2014), which filter water and thus accumulate more metals. Al^{+3} content is higher in algae than in fish, the former being indicators of contamination as a biomarker to monitor environmental pollution. The presence of five-fold Al^{+3} and Zn^{+2} higher in the fish meat from the Cachoeira river than in farmed fish can be attributed to the different degree of environmental pollution (the Cachoeira river receives effluents from domestic and industrial waste and factors linked to fish (species, sex, age). However, considering WHO (2011) weekly intake recommendations of 2 mg/Kg of this metal, it is noticeable that the amount contained in the fish from Cachoeira river exceeds this recommendation, making chronic exposure dangerous for children, the elderly, and renal disease patients (since the main route of elimination of Al^{+3} is urinary).

In the study by Abdelazim et al. (2018), exposure of tilapia to ZnO nanoparticles (1 and 2 mg/L) for two weeks resulted in several changes in the muscles of the fish at the oxidative stress level: the activity of the enzymes CAT, SOD, and GSH-Px was significantly decreased, as well as the expression of genes for those. There was also elevation of lipid peroxidation, and since these changes were prevented by vitamins C and E, the authors attributed the findings of increased oxidative stress to ZnO nanoparticles. In the study by Tawell et al. (2012), between 11-16 ppm Zn^{+2} was found in tilapia muscle, an

acceptable amount for human consumption. Dwivedi et al. (2015), on the other hand, found low level of Zn^{+2} contamination in tilapia muscle, but which slightly exceeded the limit for consumption. In both studies, Zn^{+2} was the most found metal in fish muscle, which converges with the findings of the present study. However, considering the maximum allowable limit for Zn^{+2} established by FAO/WHO (2006), which is 100 mg/Kg (ppm), it can be noticed that the muscle of tilapia from Cachoeira river exceeds it by more than three times.

Therefore, the fact that the changes were associated with the consumption of cooked fish meat in the present study supports the hypothesis of concern for the risk of chronic problems linked to toxicity along the food chain, which is the most important differentiator of the present study because it comes closest to the actual exposure to which the organisms are subjected.

Despite the initial toxicity being at the cellular level, in the long term, as the consumption of contaminated fish meat becomes more chronic, it is possible that it reaches systemic levels and thus incurs a greater insidious threat to public health, since the citizens of the municipality have already adopted the practice of fishing and consuming fish meat from the Cachoeira river. However, among the studies conducted on the toxicity of heavy metals in humans, these refer to occupational toxicity. As such, although the predictive power (in terms of extrapolation of pre-clinical findings to the risk to humans) is limited, the evidence that consuming meat from a polluted river may incur significant disruption of the oxidative system that may accumulate along the food chain must receive attention from the authorities in terms of strategic planning to sensitize the population to the existence of the risk, in addition to the adoption of measures to improve the quality of the water of the river.

V. CONCLUSION

Consumption of cooked tilapia meat from the Cachoeira river for one month promoted a significant increase of ROS in the heart, cerebellum, liver, and plasma and a significant reduction of CAT activity in the liver and SOD activity in the kidney of the animals; and these changes may be related to the higher amount of Al^{+3} and Zn^{+2} present in the fish meat from the polluted river. These data suggest that chronic consumption of fish meat contaminated by environmental pollution with heavy metals has the potential for long-term health endangering toxicity that may result in clinical presentation of pathologies, as oxidative stress causes chronic and cumulative cellular damage.

ACKNOWLEDGMENTS

This research was funded by the Brazilian Superior Level Personal Improvement Coordination (CAPES) and the Support Research Funding (FAP) from UNIVILLE.

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