Mitochondria-rich cells changes induced by nitrite exposure in tambaqui (*Colossoma macropomum* Cuvier, 1818)

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ABSTRACT

The gill mitochondria-rich cells of the juvenile Amazonian fish *Colossoma macropomum* were analyzed using light and scanning and transmission electron microscopy after 96 h exposure to 0.04 and 0.2 mM nitrite. Although the number of mitochondria-rich cells decreased significantly in the lamellar epithelium, no decrease was found in the interlamellar region of the gill filament. Nitrite exposure caused significant reduction on the apical surface area of individual mitochondria-rich cells ($p < 0.05$), with a resulting reduction of the fractional area of these cells in both the lamellar and filament epithelium. Swelling of endoplasmic reticulum cisternae, nuclear envelope and mitochondria were the main changes found in the mitochondria-rich cells. Cristae lysis and matrix vacuolization characterized the mitochondrial changes. The overall ultrastructural changes indicated cellular functional disruption caused by exposure to nitrite. The changes observed in the gill indicate that the cellular structures involved in the process of energy production become severely damaged by exposure to nitrite indicating irreversible damage conducting to cell death.

Key words: cell damage, fish, gills, morphometry, ultrastructure.

INTRODUCTION

Nitrite (NO_2^-) is an intermediate product of bacterial ammonia decomposition that occurs naturally in ecosystems. Normally, low environmental levels are maintained as a result of balanced bacteria-driven nitrification and denitrification processes

(Lewis Jr and Morris 1986, Jensen 2003). At low concentrations, NO_2^- is beneficial for fish as an exogenous reservoir for nitric oxide, which is a key signaling molecule in cardiovascular regulation (Hansen and Jensen 2010, Jensen and Hansen 2011). High concentrations are related to mass fish mortality (Svobodová et al. 2005). Some environmental conditions such as temperature and

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high levels of organic matter favor the occasional development of NO_2^- in the Amazonian environment and in fish culture systems. The active mechanism for NO_2^- uptake by the gill mitochondria-rich cells (MRCs) and its possible toxic effect on these cells were previously discussed (Jensen 2003, Williams and Eddy 1986, Gaino et al. 1984).

Nitrite enters the bloodstream via the same mechanism of Cl^- uptake on gill MRCs. Inside the fish, NO_2^- promotes methemoglobinemia, decreasing the O_2-carrying capacity of the blood, and bioconcentrates in liver, brain, muscle, and gills, disrupting its functions (Margiocco et al. 1983, Jensen 2003, Tomasso 2012). In the present, scarce information is available on the ultrastructural changes leading to cell damage under NO_2^- stress in freshwater fish. The main purpose of the study reported on herein was to evaluate the effect of NO_2^- on the distribution and ultrastructure of the branchial MRCs of an Amazonian serrasalmid species, *Colossoma macropomum* Cuvier, 1818, which is locally known as tambaqui and inhabits waters with low levels of Cl^-, low pH and, periodically hypoxic. This water-breathing species is intensively exploited in aquaculture programs but little is known about its tolerance to toxic compounds.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

This study was conducted in accordance with national and institutional guidelines for the protection of human and animal welfare. Juvenile specimens of *C. macropomum* [body mass = 73.66 ± 4.00 g, mean ± sem.], were obtained from Centro Nacional de Pesquisa e Conservação de Peixes Continentais (CEPTA, Pirassununga, Brazil, with permission from MMA/SISBIO; Protocol Num. 33918). Fish were kept indoors for at least 4 weeks in 1,000-liter holding tanks supplied with running aerated water (with a temperature of 21°C, pH of

7.4, water $PO_2 > 130$ mmHg, $[Ca^{2+}]$ of 0.043 mM; $[Na^+]$ of 0.034 mM; $[Cl^-] < 0.014$ mM; and $[NO_2^-] < 0.004$ mM). The animals were fed daily to satiation with commercial fish food pellets. Excess of food decanted at the bottom of the tanks were removed by siphoning. Feeding was suspended two days before the experiments. The photoperiod adopted was 12 h of daylight and 12 h of night throughout the acclimation and experimental periods.

NITRITE EXPOSURE

Ten fish per treatment were placed in an experimental aquarium (with a volume of 63 L) supplied with continuous aerated water from the same source as the holding water and acclimatized for 24 h prior to adding reagent-grade $NaNO_2$ (Merck, Germany) to provide the selected concentrations of 0.04 and 0.2 mM $NaNO_2$; 96 h LC_{50} $NO_2^- = 0.16$ mM according to Costa et al. (2004). The NO_2^- concentration was measured and adjusted according to Strickland and Parsons (1972). Similar tests without addition of NO_2^- were used for control purposes. Observed deaths were reported. Following the 96 h exposure to NO_2^-, the fish were removed from the test aquarium and anesthetized (0.5 % Benzocaine, Sigma-Aldrich, USA), and sacrificed with a blow to the head.

SAMPLING AND TISSUE PROCESSING

The second gill arch on the right-hand side was immediately excised and washed in a 0.9 % saline-sucrose solution. For light microscopy (LM), filaments (n = 6/fish) that were still attached to the septum of the arch were immersed in 4 % paraformaldehyde, 1 % glutaraldehyde in 0.1 M of phosphate-buffer saline (pH 7.2) fixative solution for 24 h. The samples were dehydrated and embedded in Historesin® (Leica, Heidelberg, Germany). Serial sections 1 mm thick, a constant distance of 40 mm apart, were obtained from the trailing edge to the leading edge of the filament

(longitudinal sections perpendicular to the surface of the secondary lamella). Sections were stained with PAS (periodic acid-Schiff) reaction and subsequently 0.5 % toluidine blue (Kiernan 2008) and analyzed under a BX-51 Olympus microscope equipped with a Panasonic DMC-FZ20 camera (Osaka, Japan).

For transmission electron microscopy, small fragments of filaments that contained lamellae were fixed in 2.5 % glutaraldehyde solution buffered with 0.1 M phosphate-buffer saline (pH 7.3) for 2 h. The samples were post-fixed in a 1 % OsO_4 solution, dehydrated with an acetone series, and embedded in Araldite 6005 (Ladd Research Industries, Inc., Burlington, VT, USA). Ultra-thin (60-70 nm thick) sections were contrasted with uranyl acetate and lead citrate and examined under a Philips CM 120 electron microscope. Sixteen photomicrographs from each fish were analyzed for cytological changes in MRCs, and the results were presented as semi-quantitative means of the ultrastructural changes observed.

For the scanning electron microscopy analysis, pairs of filaments attached to the septum were fixed in 2.5 % glutaraldehyde solution buffered with 0.1 M phosphate-buffer saline (pH 7.3) for 2 h. The samples were dehydrated with a graded ethanol series up to pure ethanol and then CO_2 critical-point dried. Filament pairs were glued with silver paint onto the specimen stub (the lateral side of the filaments parallel to the face plan of the stub plate), coated with gold in a vacuum sputterer and examined under a Zeiss DSM 940 scanning microscope at 25 kV.

GILL MORPHOMETRY AND CELL COUNTS

Morphometric analyses were performed on 1 mm thick serial sections stained with PAS- toluidine blue. Digitized images were analyzed using the SigmaScan software package (SYSTAT, Statistical and Graphical Software, CA, USA). During this analysis, a Merz test array (Merz 1967) was superimposed over the video monitor to ensure random orientation of the measurements on each section at a magnification of at approximately 1,400. The Merz intercepts with the borders of the lamellar epithelia and lamellar and filament epithelia were used to define the starting point for a two-points measurement [modified from (Audet and Wood 1993)]. The thickness of the lamellar and filamentar epithelium was measured from the surface in contact with the ambient water to the respective basement membrane. The thicknesses of the lamellae and filaments were measured from the external surface to the external surface of the respective region. Thirty randomly located measurements were performed per fish. The frequency of MRCs was counted on five consecutive lamellae and respective interlamellar regions of filaments on three different filaments per fish (90 measurements/fish). All cell counts and measurements were performed using a randomized blind method in which the counter did not know whether tissue sections were from NO_2^--treated experimental or untreated control animals.

Measurements of the apical surface area of MRCs were performed on the trailing edges of the filaments (10-20 lamellae distal from the septum) using scanning electron microscopy. The apical surface area was determined by tracing the cell perimeter on a calibrated digitizer tablet using the SigmaScan software package (SYSTAT, Statistical and Graphical Software, CA, USA) according to (Bindon et al. 1994). Random areas were recorded at a magnification of approximately 1,500. The apical MRC area was used to calculate the MRCs fractional surface area (FSA, i.e., the fraction of the gill filament epithelial surface that was occupied by MRCs) and the cell density according to the following equations:

STATISTICAL ANALYSES

All data are expressed as the mean ± sem (standard error of the mean) unless stated otherwise. The statistical significance between data sets was determined using one-way ANOVA followed by Bonferroni *post hoc* test with a significant level of 5 %. Statistical analyses were performed using the SigmaStat software package (SYSTAT, Statistical and Graphical Software, CA, USA).

RESULTS

Mortality after 96 h of NO_2^- exposure was 20 and 60 % (0.04 and 0.2 mM, respectively). No deaths were observed in the control group.

The morphology of gill filaments and lamellae of *C. macropomum* (Figure 1a-c) is similar to that described for other teleost species. The secondary lamellae were regularly spaced on both sides of the gill filaments and consisted of two epithelial layers of cells separated by rows of pillar cells interspersed with blood spaces. The control fish of this species exhibited numerous MRCs on their lamellae (Figure 1a). Fish exposed to 0.04 mM presented thinner and longer lamellae interspersed with protruding and swollen MRCs with varied morphologies. Pillar cells flanges were apparently retracted, thus revealing increased vessel space (Figure 1b). At a higher level of NO_2^- exposure (0.2 mM), filaments had a slender appearance. The filament epithelium was only 3-4 cells thicker (Figure 1c) and thickness of lamella was reduced in more than 30 % (Figure 1d). Following these changes, we observed a significant decrease in the thickness of epithelium of lamella, approximately 40 % for both NO_2^- exposures (Figure 1e). The thickness of filament was significantly reduced after exposure to NO_2^- (16 % and 31 %, respectively, in fish exposed to 0.04 and 0.2 mM) (Figure 1f). The filamentar epithelium was reduced by 25 % and 42 % following the same pattern previously reported (Figure 1g). Nitrite exposure reduced the frequency of MRCs on lamella approximately 51-57 % (Figure 1h), but no significant changes in the frequency of these cells on the filament (interlamellar region) were observed (Figure 1i).

Nitrite stress resulted in slender shape lamellae; more pronounced at higher concentration tested (Figure 2a-c). Nitrite exposure reduced the FSA and the apical surface area of MRCs approximately 30 % (Figure 2d-e). No change in the density of the MRCs on filaments was observed (Figure 2f).

Table I summarizes the cytological analysis of MRCs of control *C. macropomum* and fish exposed to NO_2^- for 96 h. Ultrastructure of MRCs from the control group was characterized by a high mitochondrial density in the cytoplasm and an extensive and anastomosing tubular system. The cisternae of endoplasmic reticulum (ER) were discreetly distributed throughout the cytoplasm. The mature MRC presented mitochondria with ellipsoidal shapes and extensive but not dilated cisternae of the crista. Spherical vesicles were dispersed in the cytoplasm. Nuclear euchromatin was prevalent over electron-dense heterochromatin. The large majority of MRCs was totally exposed to ambient water, but some cells remained retracted surrounded by flattened pavement cells (Figure 3a). Fish exposed to 0.04 mM (Figure 3b) exhibited MRCs with pronounced distension of the tubular system, ER and proliferation of lysosome-like bodies. The mitochondria were characterized by distention of the mitochondrial matrix, which was frequently filled with dense bodies. The nuclei displayed slight deformation of the nuclear envelope (dilation of the perinuclear space). Many cells presented a low electron-density and scarcity of organelles at the basolateral region. The apical surface of the MRCs in contact with the ambient water was frequently restricted by projections from pavement cells. Many MRCs presented cellular and organelle swelling. In fish exposed to 0.2 mM (Figure 3c), the previous related alterations were more pronounced. The cells reached the maximum
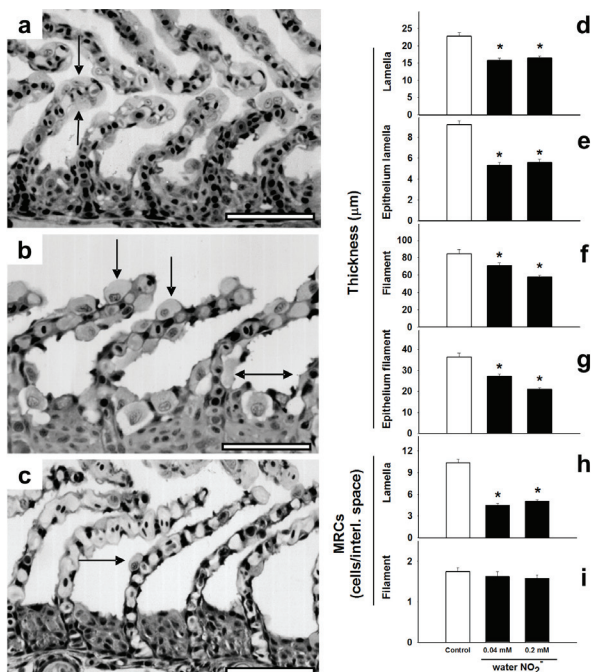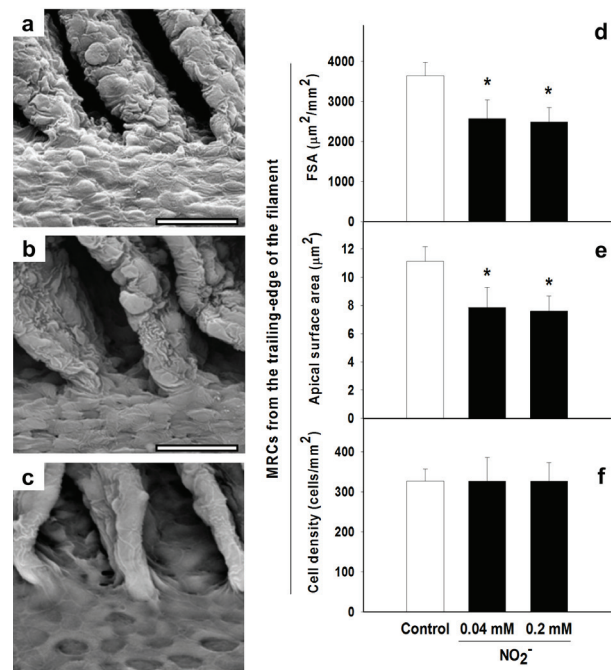
Figure 1 - Light micrographs and morphometry of gills of *C. macropomum*. **a**. Filament and lamellae from the control group exhibited numerous MRCs (arrows) and swollen and short lamellae. MRCs are easily distinguished in this resin section because of their round shapes. **b.** 0.04 mM NO_2^-: these lamellae were characterized by a gradual decrease in the number of MRCs and increased interlamellar space (double arrowhead). Some MRCs exhibit swelling and visible changes in the nuclear material. **c.** 0.2 mM NO_2^-: scarce MRCs were observed on lamellae. **d**. Thickness of lamellae; **e**. Thickness of lamellar epithelium; **f.** Thickness of filament; **g.** Thickness of filament epithelium. **h.** Frequency of MRCs on lamellae and **(i)** filament. *Significantly different (p < 0.05) from control. Scale bars = 25 μm

Figure 2 - SEM and surface measurements of gills of *C. macropomum*. **a.** Control group. **b.** 0.04 mM NO_2^-. **c.** 0.2 mM NO_2^-. Note the changes in the structure of lamellae and filament surface after exposure to NO_2^-. **d.** MRC fractional surface area. **e**. Apical surface area. **f.** MRC density. *Significantly different (p < 0.05) from control. Scale bars = 10 μm.

swelling and internally were characterized by progressive loss of organelles and membrane breakdown charactering necrosis.

DISCUSSION

Our results showed that NO_2^- exposure of *C. macropomum* promoted morphological changes in the gill epithelium and damage of MRCs. In general, these results confirmed the high sensitivity of *C. macropomum* to NO_2^- reported by Costa et al. (2004) where hemolytic anemia was the main change observed in fish blood. These morphological findings are potential inducers of severe ion and osmoregulation disturbance in freshwater fish living in ion poor water.

Effects of NO_2^- exposure on fish ionic homeostasis are relatively well-known (Jensen et al. 1987, Knudsen and Jensen 1997, Lewis Jr and Morris 1986, Martinez and Souza 2002, Svobodová et al. 2005, Williams and Eddy 1986). The MRCs are the main cell responsible for ion exchange across gill membranes and is the first target for NO_2^- action and where it accumulates and is made available to the bloodstream. In this study, MRCs were lost from lamellae and its apical surface area was reduced suggesting decreased ion uptake (Evans et al. 2005). This decrease in contact with ambient water was promoting by cytoplasmic projections of PVCs overlapping MRCs, such condition also imply reduced involvement with ionic regulation (Marshall et al. 1997).
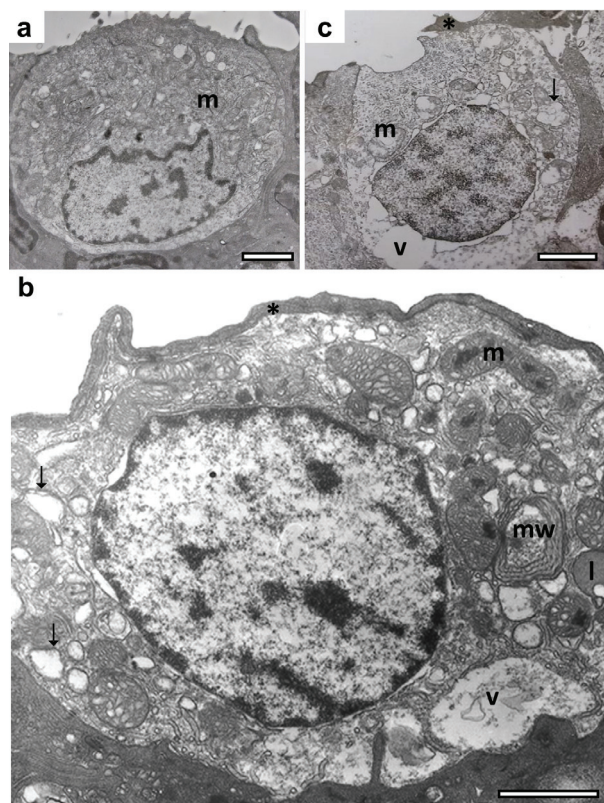
Figure 3 - TEM of MRCs from lamellae of *C. macropomum*. **a.** Control group. **b.** 0.04 mM NO_2^-. **c.** 0.2 mM NO_2^-. Hypertrophic MRCs were filled with distend and dense mitochondria (m), distend tubular system (arrow), lysosome-like bodies (l), myelin whorls (mw) and large vacuoles (v). * = extension of pavement cell. Scale bars: a, c = 1 μm; b = 5 μm.

TABLE I
Ultrastructural changes in the gill MRCs of *C. macropomum* after exposure to NO_2^- concentrations for 96 h.

Cytological Parameters	$[NO_2^-]$ (mM)		
	0.00	0.04	0.20
Nuclei			
Deformation of nuclear envelope	-	++	++++
Dilation of nuclear envelope cisternae	-	++	++++
Tubular system			
Overall amount	++	+++	++++
Dilation of cisternae	-	+++	++++
Fragmentation of cisternae	-	+++	+++
Lysosomal elements			
Overall amount	+	++	+++
Myelinated bodies	+/-	++	+++
Cytoplasmic vacuoles	++	++	+++
Mitochondria			
Overall amount	++++	+++	++
Morphological heterogeneity	-	++	+++
Formation of myelin-like whorls	-	++	+++
Vacuolization	-	++	+++
Outer membrane rupture	-	-	++

Coding: -, absent; +/-, very little developed; +, little developed; ++, moderately developed; +++, strongly developed; ++++, very strongly developed.

The high density of MRCs in the lamellar epithelium of *C. macropomum* of the control group may be due to the low ion concentration in the environmental water, which characterizes Brazil's continental waters. Several studies have shown high MRCs proliferation in fish living in ion-poor water (Perry 1997), although Fernandes and Perna-Martins (2002) noted the significantly variable number of MRCs in Brazil's freshwater fish.

The sharp reduction of MRCs in lamellar epithelium may be related to increased cell death and low cellular differentiation in this epithelium. Indeed, most of the ultrastructural changes in MRCs displayed a morphological pattern of necrosis. Previous studies have suggested that NO_2^- increases MRCs activity to maintain ion homeostasis (Gaino et al. 1984), which, in combination with the direct toxic effects of NO_2^-, contributes to reduce the cell's cycle.

Our results are, in general, congruent with those found in the ultrastructure of MRCs of *Oncorhynchus mykiss* exposed to 450 μgN-NO_2/L

(0.03 mM NO_2^-) for 72 h (Gaino et al. 1984). Furthermore, we also found signs of severe damage in mitochondria after NO_2^- exposure, evidencing clear irreversible cellular damage. Mitochondria is a target for NO_2^- binding (Shiva 2010). Nitrite binds to heme moieties of mitochondrial cytochrome components of the respiratory chain, inhibiting respiration, and stopping or reducing ATP production (Jensen et al. 2015). Mitochondrial swelling has been related to the toxic action of compounds deriving from NO_2^- in rat liver hepatocytes (Rusu et al. 1979).

Cell and organelle swelling, as reported in our study, possibly reflect the inhibition of Na^+/K^+ ATPase, promoting in cell K^+ efflux and Na^+ uptake, as previously reported in *C. carpio* (Knudsen and Jensen 1997, Jensen et al. 1987). In addition to osmotic changes, NO_2^- at high concentrations in tissue causes an excess of NO generation resulting in excessive induction of iron-nitrosylation, nitrosation of cellular thiols and amines, exacerbating NO_2^- toxicity and interfering with the functioning of many key proteins (Jensen et al. 2015).

In our study, we present evidence that cell death by non-apoptotic program (necrosis) was prevalent and is not morphologically different from that described for other species exposed to toxic substances (Mallatt 1985) or following transfer from fresh water to seawater (Bonga and Van der Meij 1989). Evidences indicate that necrosis is the main cell death program triggered when ATP levels drop rapidly after NO_2^- stress (Leist et al. 1997, Brown and Borutaite 2002, Leist et al. 1999). In opposition to other studies, few cells were demised from epithelia by apoptosis, however, this cell death program has been reported after NO_2^- intoxication in fish (Jia et al. 2015, Sun et al. 2014). The prevalence of necrotic MRCs may evidence variability in energy use of cells or may indicate different levels of mitochondrial damage which

may trigger one or the other mechanism of cell death.

In conclusion, MRCs are particularly affected by NO_2^- exposure. The changes observed in this study indicate that NO_2^- is toxic to *C. macropomum* and has a potential to affect important physiological process by disrupting the MRC ultrastructure and its distribution along the branchial epithelium. The changes observed in the gill indicate that the cellular structures involved in the process of energy production become severely damaged by exposure to NO_2^- indicating irreversible damage resulting in cell death.

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