

Evaluation of mitochondrial membrane potential and DNA integrity in Catfish *Pseudoplatystoma magdaleniatum*, when exposed for prolonged times to different concentrations of ibuprofen.

Sara E. Gallego Ríos (✉ sara.gallego@udea.edu.co)

Universidad de Antioquia <https://orcid.org/0000-0002-5143-1869>

Gustavo A. Peñuela

Universidad de Antioquia

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Abstract

There are few studies to date that determine the effects of ibuprofen on mitochondrial membrane potential ($\Delta\Psi$ M) and DNA integrity in neotropical fish. The objective of this study is to determine if four months' exposure to ibuprofen in different concentrations (25 and 50 $\mu\text{g/L}$) produces effects on $\Delta\Psi$ M and alters the integrity of DNA in striped catfish *Pseudoplatystoma magdaleniatum*. For this study, the fish were placed in tanks with water at constant concentrations of 0 (control), 25, and 50 $\mu\text{g/L}$ of ibuprofen for four months. Subsequently, blood samples were taken for analysis of $\Delta\Psi$ M and DNA integrity, using a flow cytometer LSRFortessa BD Biosciences. After four months of exposure to ibuprofen at different concentrations, the results showed no increase in Low $\Delta\Psi$ M, indicating that there are no alterations in the mitochondrial membrane potential. On the other hand, the percentages of DNA damage were below 0.39, which indicates that there were no alterations in DNA integrity. It is possible that under the conditions in which this study was conducted (ibuprofen levels, exposure time), they are not sufficient to demonstrate the effects caused by this drug. Higher ibuprofen levels and/or longer exposures may be required to determine alteration in $\Delta\Psi$ M and DNA integrity. Flow cytometric analysis for these types of samples is a fast, specific, and reliable technique, compared to traditional methods.

1. Introduction

The function of pharmaceuticals is as medicines or to improve the quality of daily life (Giang et al. 2018; Aguilar-Romero et al. 2020). However, their presence in aquatic environments and their possible harmful effects on aquatic organisms have caused concern in recent years (Liu et al. 2020). The main entry routes for pharmaceuticals to aquatic environments are wastewater treatment plants due to their incomplete disposal (Montagner and Jardim 2011; Giang et al. 2018; Meijide et al. 2018; García-Camero et al. 2019; Aguilar-Romero et al. 2020), and to a lesser extent, the combination of untreated rivers and runoff waters (González-Mira et al. 2016), aquaculture, and pharmaceutical manufacturing sites (Liu et al. 2020). Conventional wastewater treatment plants are not designed to eliminate most pharmaceutical products (Tiedeken et al. 2017), most of them are also persistent due to their continuous diffusion in the aquatic environment, which can remain dissolved in the water column or accumulate in sediments (González-Mira et al. 2016). Most of these compounds and their metabolites are biologically active (Sathishkumar et al. 2020) and can cause alterations in aquatic organisms exposed for long periods, causing endocrine alterations, genotoxicity, carcinogenicity, and fetal malformations, among others (Giang et al. 2019; Liu et al. 2020)

Ibuprofen is a non-selective non-steroidal anti-inflammatory drug (NSAID), this drug reversibly inhibits the synthesis of prostanoids (prostaglandins, prostacyclins, and thromboxanes) (González-Mira et al. 2016), by non-selectively inhibiting cyclooxygenase 1 and 2 enzymes and blocking the synthesis of prostaglandins and thromboxanes (Motov et al. 2020); Ibuprofen interferes with the cyclooxygenase pathway, decreasing the catalysis of prostaglandin biosynthesis from arachidonic acid (Parolini 2020). This drug can cause alterations in reproduction and development (Xia et al. 2017), oxidative stress, hematological changes, and DNA damage in fish (Mathias et al. 2018).

The striped catfish *Pseudoplatystoma magdaleniatum* is an endemic species and the second most commercially important in the Colombian fishery. However, it is among the critically endangered species (Mojica et al. 2012), mainly due to habitat degradation, embalming of rivers, overfishing, deforestation, and organic and inorganic contamination (Mojica et al. 2016; Herrera-Cruz et al. 2019). This fish inhabits the Magdalena and

Cauca basins, the main rivers of Colombia; historically, these basins have presented unsolved environmental problems, derived from deforestation, erosion, and contamination by solid and liquid waste (Galvis and Mojica 2007; Noreña-Ramirez et al. 2012; Zapata et al. 2015; Tejada-Benítez et al. 2018). This threatens both the economy of the communities where the catfish live, where it is a main source of work, income, and their food security as a source of food (Friedrich-Ebert-Stiftung and Foro Nacional Ambiental 2015).

The Magdalena river basin is the main recipient of domestic wastewater, as well as contaminated water derived from pesticides used in crops, illegal gold extraction, and some industries such as oil refining, tanneries (Tejada-Benítez et al. 2018). The investigations carried out in the Magdalena river basin have been carried out mainly in the analysis of heavy metals, leaving aside the investigations for the determination of some pollutants such as pharmaceutical products in the Magdalena and Cauca rivers (Noreña-Ramirez et al. 2012; Tejada-Benitez et al. 2016; Tejada-Benítez et al. 2018). The main source of contamination in the Magdalena and Cauca basins is wastewater from the main cities Bogotá, Medellín, Cali, and Barranquilla (Galvis and Mojica 2007; Tejada-Benitez et al. 2016). In these cities, some research has been carried out on the content of PPCPs in the waters, finding that the greatest contribution of pharmaceutical products is non-steroidal anti-inflammatory drugs, anticonvulsants, and antibiotics (Gracia-Lor et al. 2012; Hernández et al. 2015; Aristizabal-Ciro et al. 2017; Bedoya-Ríos et al. 2018; Arias 2019; Pemberthy et al. 2020).

Several studies have detected concentrations of ibuprofen in tributaries and effluents of wastewater treatment plants, surface waters, drinking water, sludge, and hospital effluents. Ibuprofen concentrations have been reported around the world in the range of 0.001 and 75.8 $\mu\text{g} / \text{L}$. (Gutiérrez-Noya et al. 2020). Ibuprofen may be present in the Magdalena river basin, causing alterations in the catfish that inhabit these areas and possibly being one of the causes of their decrease in the basin. The determination of the possible alterations in the mitochondrial membrane potential ($\Delta\Psi\text{M}$) and the integrity of the DNA can be determined by flow cytometry, since it is a fast and reliable technique to quantify and characterize some cell populations, allowing the evaluation of processes immunopathological in fish (Alzamora-Gonzales et al. 2015).

The $\Delta\Psi\text{M}$ regulates the synthesis of adenosine-tri-phosphate (ATP), the production of reactive oxygen species (ROS), the sequestration of calcium in the mitochondria, the import of mitochondrial proteins, and the dynamics of the mitochondrial (Luna-Ortiz et al. 2013; Zorova et al. 2018; Restrepo et al. 2019). $\Delta\Psi\text{M}$ is important for many mitochondrial processes and is related to mitochondrial and cellular health (Allauca et al. 2019).

Because ibuprofen is one of the most widely consumed drugs in the world (Ngo and Bajaj 2020), it is possible that it is present in the main rivers of Colombia and is one of the possible causes of the decline of striped catfish *P. magdaleniatum* in the last four decades. For this, a controlled experimental study was carried out for four months with different doses (0, 25, 50 $\mu\text{g}/\text{L}$) of ibuprofen in males and females of *P. magdaleniatum*, determining the alterations in the mitochondrial membrane potential and the integrity of the DNA, by flow cytometry.

2. Materials And Methods

2.1. Fish Husbandry

The striped catfish *Pseudoplatystoma magdaleniatum* (Siluriformes: Pimelodidae) is manifested through sexual dimorphism, reproductive migrations with temporality for spawning (Arce et al. 2014), and not possessing scales. The catfish is nocturnal, feeds on fish, some arthropods, and seeds (Santamaría Merchán 2013).

All the fish were caught in the Cauca River, Colombia. Sexually mature striped catfish, *P. magdaleniatum* were used per experimental tank, in which they were divided into males and females (three male and three female catfish were distributed in individual tanks) with average weights and lengths of 1.86 ± 0.49 kg and 61.14 ± 4.76 cm for males and 2.07 ± 0.64 kg and 63.86 ± 6.01 cm for females. All experimentation was carried out at the Fish Culture Research Institute of the University of Córdoba (CINPIC) located in Montería, in the department of Córdoba. The fish were acclimatized for two months in tanks of 3250 m^3 , with a 12/12 photoperiod throughout the year.

The fish were fed with live feed *Astyanax sp* (fed on demand), grown in fish farming. To guarantee the quality of the water in the ponds, Table 1, weekly using a portable multiparameter (HACH, Sension + MM15, USA): dissolved oxygen, temperature, pH and percentage of oxygen saturation; total alkalinity (Rice et al. 2017a); total hardness (Standard Methods 2340 C) (Rice et al. 2017b); and volatile solids and fixed solids (Standard Methods 2540 E) (Rice et al. 2017c); ammonia nitrogen and ammonia using a kit (API, USA).

Table 1
Water quality of the experimental units.

Month	Temperature (°C)	Oxygen saturation (%)	pH	Ammonium (NH ₃ /NH ₄ ⁺)	Total alkalinity (mg/L CaCO ₃)	Total hardness (mg/L CaCO ₃)	Fixed solids (mg/L)
1	29.31 ± 0.89	86.36 ± 3.89	7.60 ± 0.00	0.35 ± 0.14	64.95 ± 4.88	67.57 ± 3.21	208.5 ± 2.12
2	28.54 ± 0.38	91.85 ± 4.25	7.83 ± 0.20	0.42 ± 0.30	68.30 ± 3.68	72.78 ± 6.16	213 ± 9.90
3	28.55 ± 0.36	89.31 ± 5.89	7.93 ± 0.21	0.46 ± 0.29	69.95 ± 5.59	77.26 ± 1.48	214 ± 1.41
4	28.98 ± 0.43	91.06 ± 2.33	7.80 ± 0.00	0.42 ± 0.13	67.55 ± 5.30	75.60 ± 6.14	212 ± 5.66
Values are expressed as mean \pm SD.							
SD standard deviation.							

These species were caught with the authorizations required by current regulations, Framework Permit issued by the Autoridad Colombiana de Licencias Ambientales (ANLA) in Resolution 1461 of December 3, 2014. The number of samples was determined based on the considerations of the Ethics Committee for Animal Experimentation of the Universidad de Antioquia, with Act 89 of May 29, 2014, in which the minimum use of fish is recommended for the extraction, to minimize the impact on the capture of these fish, which have been devastated in the last decades. All animal experiments were by Directive 2010/63/EU of the European Parliament (Official Journal of the European Union 2010).

2.2. Experimental design

The fish caught for the experimentation are fish of sexual maturity size, however, the breeding season waited; the experiment lasted four continuous months, the time necessary for this species to develop its gametes (Palacio 2009; Arce et al. 2014). The experimentation was carried out with three independent experiments, the fish were divided into three groups according to their exposure: 0 control, 25 and 50 µg/L, the control group (0 µg/L) always remained below the limit of detection. Due to the photochemical degradation and absorption of ibuprofen by the fish, 50% of the water in the tanks was replaced weekly and the ibuprofen concentrations were readjusted in each tank. Water samples were taken in amber glass containers, kept at 4–6°C for 24 hours. Ibuprofen was quantified using an ultra-performance liquid chromatography-tandem mass spectrometer (UHPLC-MS/MS) following the modified EPA method 1694 (Environmental Protection Agency (EPA) et al. 2007).

The ibuprofen concentrations for the experiment were determined taking into account some environmental concentrations present in the tributaries (< 0.984–6.328 µg/L) and effluents (< 0.065–0.491 µg/L) of wastewater treatment plants (Kasprzyk-Hordern et al. 2009) and some rivers in the United Kingdom (1.681–33.764 µg/L (Petrie et al. 2014)). Also, the sublethal LC50 (1/10 of LC50) of some similar species, 14.2 µg/L, was taken into account (Saravanan et al. 2012). Taking into account the levels of this medically in the water and an estimate of the sublethal dose, a concentration of 25 µg/L and 50 µg/L was determined to evaluate the possible effects.

The concentrations of ibuprofen added were determined taking into account environmental concentrations present in the tributaries (< 0.984–6.328 µg/L) and effluents (< 0.065–0.491 µg/L) of wastewater treatment plants (Kasprzyk-Hordern et al. 2009) and some rivers in the United Kingdom (1,681 – 33,764 µg/L (Petrie et al. 2014)). As well as sublethal LC50 (1/10 of LC 50) of some similar species 14.2 mg/L (Saravanan et al. 2012). Taking into account the ibuprofen levels in the water and an estimate of the sublethal dose, a concentration of 25 µg/L and 50 µg/L was determined to evaluate the possible effects on mitochondrial membrane potential and DNA integrity.

2.3. Blood Sampling

Blood samples were taken 14 days after the addition of ibuprofen and after four months. Blood samples were collected by a direct puncture in the tail vein with the help of a vacutainer; the tubes in which the samples were collected contained EDTA K2 anticoagulants. Blood samples were taken for each treatment and all samples were processed separately. Blood was drawn from each fish and kept for 1 hour at 4–6 ° C and in the absence of ultraviolet light until reaching the laboratory for analysis.

2.4. Determination of Mitochondrial Membrane Potential ($\Delta\Psi_M$)

For the determination of $\Delta\Psi_M$ in the blood samples, 3,3'-dihexyloxycarbocyanin iodide (DiOC₆, Molecular Probes by Life Technologies, Thermo Fisher Scientific) was used. This fluorescent dye is used for mitochondrial staining under the influence of the permeability transition (Rojas et al. 2000; Rieger et al. 2011). For analysis, a tube was used to add the DiOC₆ in phosphate buffer (PBS) to a final concentration of 800 nM, then 10 µL of blood was added. Subsequently, to stain the cells to simultaneously assess their viability, 1 µg /mL of propidium iodide (PI, Thermo Fisher Scientific) was added to each tube. The samples were incubated for 30 minutes and the $\Delta\Psi_M$ was measured by flow cytometry (LSRFortessa, BD Biosciences). Subsequently separated in three ways according to the intensity of the DiOC₆ in the Moflo XDP using a 70 µm nozzle, at a frequency of 100 thousand

Hz, with a minimum efficiency of 98 for each of the three separations ways. The temporality analyzes had been made on the high uptake and intermediate uptake cells of DiOC₆, Fig. 1.

The $\Delta\Psi$ M of the blood was assessed with an adapted version of the protocol described by Zamzami et al. (1996). A polystyrene tube was used to deposit 3.3'-dihexyloxacarbocyanine iodide (DiOC₆, Molecular Probes) in PBS at a final concentration of 80 nM and 7-aminoactinomycin D (7-AAD, Molecular Probes) at a final concentration of 2 μ g/ml. The compounds were pipetted, and then, 10 μ l of blood were added, the final volume of the reaction was 300 μ l. Subsequently, to stain the cells to simultaneously evaluate their viability, a final concentration of 1 mg/ml of propidium iodide (PI) (Molecular Probes, USA) was added. The samples were incubated for 30 min, and $\Delta\Psi$ M was measured using flow cytometry (LSRFortessa™, BD Biosciences). The samples were excited using a 488 nm solid phase laser, and fluorescence from DiOC₆ and 7-AAD has detected at 530/30 nm and 630/30 nm, respectively. The flow cytometry data were analyzed using FlowJo version 7.6.2 (FlowJo, LLC, USA) software.

2.5. Determination of DNA Integrity

DNA integrity was evaluated by PI staining. Cells were fixed with 300 μ L of 70% ethanol (Merck, Germany) prepared in PBS (pH 7.4) for 12 hours at 4°C. The samples were centrifuged (500 g, 5 minutes, 4°C) and the resulting granules were washed twice more with 3.0 mL of PBS. Cells were stained with PI 1 μ g/mL in PBS with 0.37% w / v EDTA, 0.01% v / v of Triton X-100 and 200 U/mL of RNase A (Sigma-Aldrich). The samples were incubated for 30 minutes and DNA integrity was measured by flow cytometry (LSRFortessa, BD Biosciences) (Restrepo et al. 2019).

2.6. Analysis of Flow Cytometry

A flow cytometer with LSRFortessa equipment (BD Biosciences, San Jose, CA), using FlowJo 10.6 software (Tree Star Inc., Ashland, Oregon, United States) was used to perform the analyses. The determination of mitochondrial membrane damage was performed by excluding aggregates by selecting the cell population of interest after contrasting the size (FSH) and granularity (SSC) to select unique events. After performing this exclusion, DiOC₆-positive cells were compared with propidium iodide (PI)-negative cells, differentiating between the populations of erythrocytes and leukocytes, Fig. 2.

For the determination of DNA integrity, an exclusion of aggregates was also performed by selecting the cell population of interest after contrasting the size (FSH) and granularity (SSC), to select the unique events. Once the unique events were selected, employing PI-A and PI-W, the single cells were selected to determine the DNA integrity employing the histogram, Fig. 3.

2.7. Statistical Analysis

Statistical analysis was performed using Statgraphics Centurion XVII (StatPoint Inc., USA). The evaluation of the normality of the continuous variables was performed via the Shapiro-Wilk test. Nonparametric statistics were applied to those variables that were not normally distributed. An analysis of variance (ANOVA) was used to evaluate the existence of significant differences between $\Delta\Psi$ M and DNA integrity. If this gave a statistically significant difference, a post-ANOVA by the least significant difference test (LSD-Fisher) was used. Statistical differences for PI+ and $\Delta\Psi$ M were analyzed by two-way analysis of variance (ANOVA) with exposure time,

concentration, and "time x concentration" interaction as variables. For all statistical analyzes, the significance criterion was established at $p < 0.05$.

3. Results And Discussion

Various studies have been carried out in fish on the alterations that ibuprofen present in the waters can cause. Finding increased glutathione-S-transferase activity in the kidney, reduced glutathione peroxidase activity, decreased white blood cell count, causing nephrotoxicity and immunosuppressive effect (Mathias et al. 2018), increased cardiac output in embryos, decreased cell density (Zhang et al. 2019), and a significant reduction in the hatching rate (Xia et al. 2017). So far, no publications have been found on the possible alterations on the integrity of the DNA in *P. magdaleniatum* caused by the presence of ibuprofen in the waters.

For this study, blood samples of striped catfish, *Pseudoplatystoma magdaleniatum*, collected from fish exposed to concentrations of 25 $\mu\text{g/L}$ and 50 $\mu\text{g/L}$ of ibuprofen for four consecutive months, and control fish without exposure, were analyzed. Environmental concentrations were used, as in some of the studies mentioned above. An analysis of $\Delta\Psi\text{M}$ was performed, as an indicator of cell viability. This reflects the hydrogen pumping through the internal membrane in the electron transport and oxidative phosphorylation processes. These processes are necessary for the production of ATP, which means that mitochondrial dysfunction is closely related to an alteration in the membrane potential that would cause a decrease in the production of ATP (Padmini and Usha Rani 2011).

We analyzed the variations between the $\Delta\Psi\text{M}$ and cells with damage to the cell membrane, positive for PI. Table 2 shows the results for a two-way analysis of variance (ANOVA), where it is observed that there is no statistically significant difference in the PI + samples between the different treatments, nor in the comparisons concerning the four months of exposure. For the $\Delta\Psi\text{M}$ there is no statistically significant difference between the High $\Delta\Psi\text{M}$, however, comparing the $\Delta\Psi\text{M}$ between the treatments and after exposure, the Medium $\Delta\Psi\text{M}$ presents statistically significant differences ($p < 0.05$) with an increase in the percentage after four months of treatment, as well as a decrease in the percentages of Low $\Delta\Psi\text{M}$ after four months of exposure to ibuprofen. Despite this difference between Medium and Low $\Delta\Psi\text{M}$, there was no decrease in $\Delta\Psi\text{M}$ after four months of treatment with ibuprofen, therefore, this drug can be indicated at concentrations of 25 and 50 $\mu\text{g/L}$, for four months, not produces loss of mitochondrial function (Blanco and López-Armada 2005).

Table 2
 Variations in mitochondrial membrane potential ($\Delta\Psi$ M) and PI+, concerning exposure time and concentrations of ibuprofen to which *P. magdaleniatum* were exposed.

Parameter	Source of variation		
	Time	Concentration	Interactions
	p-value	p-value	p-value
PI +	0.96	0.32	0.79
High $\Delta\Psi$ M	0.93	0.83	0.23
Medium $\Delta\Psi$ M	0.001*	0.43	0.36
Low $\Delta\Psi$ M	0.01*	0.54	0.43
p < 0.05, ANOVA for exposure time and concentration.			
*Significant factor.			

Table 3 shows the analysis of $\Delta\Psi$ M, the median fluorescence intensity, determined by sex and ibuprofen concentration in the different analysis times. For females exposed to 25 $\mu\text{g/L}$ of ibuprofen and presenting ratios 0.75 and 076 (High $\Delta\Psi$ M and Medium $\Delta\Psi$ M, respectively), it is indicated that at the time of the assay their leukocytes had a less mitochondrial function at time zero. However, females exposed to 50 $\mu\text{g/L}$ and presenting a ratio of 1.63 had mitochondrial hyperactivity at time zero. Both events are due to a type of stress that can be interpreted respectively as depolarization and hyperpolarization. Meanwhile, the analyses performed after four months of exposure to this ibuprofen show, for males exposed to a concentration of 50 $\mu\text{g/L}$ with ratios of 0.68 and 0.70 (High $\Delta\Psi$ M and Medium $\Delta\Psi$ M, respectively), that 32% and 30% of their leukocytes present lower DIOC₆ uptake than the control at the time of the test. For those that have a ratio above 1 (High $\Delta\Psi$ M), there are 12, 28, and 34% of leukocytes with some hyperpolarization and, as these do not exceed 1.5, this may be due to the fluctuations of the test.

Table 3

Percentage of mitochondrial membrane potential ($\Delta\Psi$ M), median fluorescence intensity, and ratio, related by sex and ibuprofen concentration in the different analysis times applied to *P. magdaleniatum*.

Sex	Concentration ($\mu\text{g/L}$)	Time zero								
		High $\Delta\Psi$ M (%)	MFI High	Ratio	Medium $\Delta\Psi$ M (%)	MFI Medium	Ratio	Low $\Delta\Psi$ M (%)	MFI Low	Ratio
	0 control	12.0 \pm 4.8 ab	3561	1.00	30.0 \pm 6.2 ab	1972	1.00	2.9 \pm 3.7 ab	1076	1.00
Female	25	9.1 \pm 2.9 a	3352	0.76	22.6 \pm 5.9 a	2210	0.75	1.1 \pm 1.0 a	568	0.36
Male		10.9 \pm 1.0 ab	3547	0.91	32.9 \pm 7.3 ab	1957	1.10	1.2 \pm 0.8 b	1154	0.42
Female	50	19.6 \pm 12.4 b	3613	1.63	17.8 \pm 3.3 a	2250.5	0.59	0.5 \pm 0.2 a	525.5	0.00
Male		13.1 \pm 6.0 ab	3497	1.09	37.2 \pm 7.1 abc	1777	1.24	4.4 \pm 3.6 ab	1181	1.51
Sex	Concentration ($\mu\text{g/L}$)	Four months								
		High $\Delta\Psi$ M (%)	MFI High	Ratio	Medium $\Delta\Psi$ M (%)	MFI Medium	Ratio	Low $\Delta\Psi$ M (%)	MFI Low	Ratio
	0 control	11.8 \pm 1.6 ab	4200	1.00	75.7 \pm 3.1 d	2070	1.00	0.3 \pm 0.4 a	686	1.00
Female	25	13.2 \pm 4.5 ab	3670	1.12	74.7 \pm 3.6 d	1995	0.99	0.5 \pm 0.7 a	710	1.52
Male		15.1 \pm 6.0 ab	4242	1.28	73.6 \pm 2.9 d	2340	0.97	0.1 \pm 0.1 a	523	0.26

Mean \pm SD.

High $\Delta\Psi$ M: samples with high mitochondrial membrane potential.

Medium $\Delta\Psi$ M: samples with medium mitochondrial membrane potential.

Low $\Delta\Psi$ M: samples with low mitochondrial membrane potential.

MFI: median fluorescence intensity.

Different lowercase letters in the columns indicate statistically significant differences ($p < 0.05$).

Female	50	15.9 ± 11.3 ab	3941	1.34	69.1 ± 11.4 ^{cd}	2418.5	0.91	0.0 ± 0.0 ab	396	0.13
Male		8.0 ± 4.8 a	4425	0.68	53.1 ± 37.8 ^{bcd}	2322	0.70	0.1 ± 0.0 a	499	0.16
Mean ± SD.										
High $\Delta\Psi$ M: samples with high mitochondrial membrane potential.										
Medium $\Delta\Psi$ M: samples with medium mitochondrial membrane potential.										
Low $\Delta\Psi$ M: samples with low mitochondrial membrane potential.										
MFI: median fluorescence intensity.										
Different lowercase letters in the columns indicate statistically significant differences ($p < 0.05$).										

So far, no comparable results have been found where the analysis for $\Delta\Psi$ M in blood samples is performed by flow cytometry. However, the determination of $\Delta\Psi$ M by flow cytometry is suggested as a biomarker due to its higher specificity and quick quantitative assessment of the possible risk of exposure to this type of pharmaceutical (Padmini and Usha Rani 2011).

Table 4 shows the integrity of the DNA, the results for time zero and after four months of exposure to ibuprofen, do not present statistically significant difference between the different treatments, and no effect on DNA is evidenced, since, after four months of exposure to ibuprofen, the average for males and females is 92.30% for 2n, which indicates that the vast majority do not present fragmentation in nonfragmented DNA and only 0.32% have some type of DNA damage.

Table 4
DNA integrity, related by sex and ibuprofen concentration at different times of experimentation in *P. magdaleniatum*.

Sex	Concentration (µg/L)	Time zero			
		2n (%)	4n (%)	< 2n (%)	Linearity
	0 control	95.300 ± 1.908	3.880 ± 1.600	0.310 ± 0.105	1.972 ± 0.014
Female	25	94.800 ± 0.700	4.527 ± 0.583	0.087 ± 0.064	1.994 ± 0.004
Male		94.333 ± 1.950	4.860 ± 1.669	0.240 ± 0.225	1.962 ± 0.019
Female	50	95.750 ± 0.212	3.560 ± 0.113	0.360 ± 0.014	1.985 ± 0.007
Male		95.600 ± 1.058	3.590 ± 0.670	0.321 ± 0.279	1.970 ± 0.022
Sex	Concentration (µg/L)	Four months			
		2n (%)	4n (%)	< 2n (%)	Linearity
	0 control	91.300 ± 0.100	6.950 ± 0.262	0.395 ± 0.114	1.928 ± 0.008
Female	25	93.233 ± 2.139	5.093 ± 2.169	0.301 ± 0.033	1.927 ± 0.020
Male		91.567 ± 1.210	6.540 ± 0.920	0.317 ± 0.035	1.930 ± 0.010
Female	50	92.650 ± 0.778	6.135 ± 0.573	0.245 ± 0.078	1.928 ± 0.008
Male		92.767 ± 2.250	5.647 ± 2.358	0.301 ± 0.092	1.940 ± 0.022
Mean ± SD.					
< 2n: DNA damage, fragmented. 2n: DNA not fragmented. 4n: Somatic cells.					

Comparing these results with other studies where DNA integrity is determined by comet assay, ibuprofen alterations were evident in monocytic cells of *Hoplias malabaricus* (Ribas et al. 2014); in *Rhamdia quelen* with ibuprofen exposure of 66.40 ng/L, where a statistically significant DNA loss was obtained after 5 and 28 days of exposure of 22.74–34.32% (Rocco et al. 2010); and in *Oreochromis niloticus*, where exposure to 300 ng/L ibuprofen caused genotoxic effects in both acute (48 h) and subchronic (10 days) exposure (Ragugnetti et al. 2011). For this study, no hypodiploid cells were evident, nor was there any loss of linearity that could be interpreted as being from DNA. It is probable that, at the concentrations at which the analysis was performed, this pharmaceutical will not cause DNA damage.

4. Conclusions

This study is one of the first to analyze alterations in the mitochondrial membrane potential and DNA integrity using flow cytometry. Catfish *Pseudoplatystoma magdaleniatum* were exposed to different concentrations (0, 25, and 50 µg/L) of ibuprofen for four months. The results for the $\Delta\Psi$ M showed a statistically significant

difference for the medium and Low $\Delta\Psi_M$, but without an increase in the Low $\Delta\Psi_M$, which indicates at these concentration levels and exposure time, there is no loss of mitochondrial function, caused by ibuprofen. There were no alterations in the integrity of the DNA, the percentages of DNA without fragmentation were higher than 90% in all sexes, connections, and exposure times. More research is needed at different levels of ibuprofen concentration, and longer exposure times; since under the conditions of this study, it was not possible to demonstrate the effects caused by ibuprofen on $\Delta\Psi_M$ and DNA integrity.

Declarations

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Conflict of Interest: The authors declare that they have no conflict of interest.

Author contributions: Sara E. Gallego R, performed activities related to Methodology, Formal Analysis, Investigation, Writing - Original Draft. Gustavo A. Peñuela was involved in the Funding Acquisition, Resources, Conceptualization, Supervision, and Project Administration. All authors contributed to writing the manuscript.

Ethical approval: These species were caught with the authorizations required by current regulations, Framework Permit issued by the Autoridad Colombiana de Licencias Ambientales (ANLA) in Resolution 1461 of December 3, 2014. All animal experiments were following Directive 2010/63/EU of the European Parliament and approved by the Ethics Committee for Animal Experimentation of the Universidad de Antioquia, with Act 89 of May 29, 2014.

Availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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