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Alterations to DNA, apoptosis and oxidative damage induced by sucralose in blood cells of *Cyprinus carpio*

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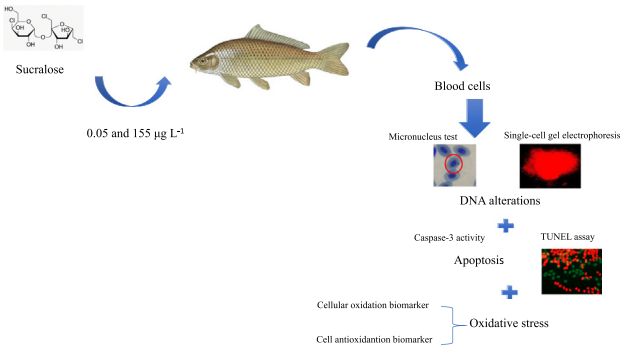
HIGHLIGHTS

- Genetic alterations and apoptosis were identified by exposure to sucralose in carp.
- Genotoxicity and cytotoxicity were identified at 0.05 and 155 μg L^{-1} of sucralose.
- Micronuclei test and the comet assay showed genetic alterations by sucralose.
- Caspase-3 and TUNEL showed that sucralose induces apoptosis.

GRAPHICAL ABSTRACT



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ABSTRACT

Sucralose (SUC) is an organochlorine that is used as a common sweetener in different dietary products around the world. Its extended use and production have led to this product is released into the environment in concentrations ranging from ng L^{-1} to μg L^{-1} in surface waters, groundwaters, wastewater treatment plants and ocean waters. A previous study carried out by our research team demonstrated that SUC is capable of inducing oxidative stress in *Cyprinus carpio* at environmentally-relevant concentrations. The aim of this study was to evaluate if SUC was capable of inducing alterations to DNA, apoptosis, and oxidative damage in the blood cells of *C. carpio*. Carps were exposed to two environmentally-relevant concentrations (0.05 and 155 μg L^{-1}) of SUC, and the following biomarkers were determined: comet assay, micronucleus test (MN), caspase-3 activity, TUNEL assay, hydroperoxide content, lipid peroxidation level, protein carbonyl content and superoxide dismutase and catalase activities. Results obtained showed that SUC is capable of inducing DNA damage. A maximum increase of 35% and 23% were observed for c1 and c2, respectively in the comet assay; increases of 586% and 507.7% for c1 and c2, respectively, were found at 72 h through the MN test. The activity of caspase-3 showed a greater response for c1 and c2 at 96 h, with 271% and 493.5%, respectively. TUNEL assay also showed the highest response at 96 h, with 51.8 for c1 and 72.9 for c2; c1 y c2 were able to induce oxidative stress with the highest expression at 72 h. A correlation between DNA damage biomarkers, apoptosis and plasma levels of SUC in both concentrations

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were observed. With the data obtained, we can conclude that SUC, at environmentally-relevant concentrations, was capable of generating DNA alterations, apoptosis and oxidative stress in blood cells in common carp.

1. Introduction

Sweeteners are defined as food additives and are used to confer a sweet taste to foods, drinks, medication and health products such as toothpaste, are synthetically-elaborated compounds, leaving common sugar as the most sweet and a lesser energy supply (García-Almeida et al., 2013). These products are employed for those who are overweight, in the treatment of obesity, maintaining body weight, control of diabetes and to prevent cavities (Calzada-León et al., 2013). The artificial sweeteners most employed globally are: aspartame, neotame, acesulfame-K, saccharin, cyclamate, sucralose (SUC) and neohespiridin dihydrochalcone: its high production and consumption by people all over the world makes it possible for these products to be released into water bodies, mainly through municipal and industrial effluents. Recently, these products have been catalogued as emerging contaminants (Kokotou et al., 2012). Of the aforementioned, only acesulfame-K, saccharin, cyclamate and SUC have been studied in water bodies (Lange et al., 2012). In literature, there are reports that refer to the fact that SUC enters wastewater treatment plants, without achieving its removal and favoring its presence in water bodies (Houtman, 2010). The occurrence of this sweetener has been evidenced in different studies. For example, at the discharge point of a wastewater treatment plant, 11 μg L^{-1} were detected; in surface waters ≤3.6 μg L^{-1} were found (Brorström-Lundén et al., 2008). In Netherlands groundwaters concentrations of 24 μg L^{-1} were found (Van Stempvoort et al., 2011). In 2009, concentrations of SUC in ocean waters from North Carolina to Florida in intervals of 67–392 ng L^{-1} were identified (Mead et al., 2009). In surface water effluents, in concentrations of ≤2.5 μg L^{-1} (Ferrer and Thurman, 2010; Loos et al., 2009; Neset et al., 2010; Scheurer et al., 2009; Torres et al., 2011), in rivers of 27 countries of Europe, concentrations above 1.0 μg L^{-1} were observed (Richardson, 2009). More recently, Arbeláez et al. (2015) found concentrations of 0.05 and 155 μg L^{-1} in Spain river. Respect to the toxic effects of SUC, the information about toxicity is limited; however, some data reported in international literature concerning the toxicity of this sweetener are the following: Lillicrap et al. (2011) evaluated the bioaccumulation capacity of SUC in algae (*Pseudokirchneriella subcapitata*), the crustacean (*Daphnia magna*) and the zebra fish (*Danio rerio*) at concentrations of 10 and 100 mg L^{-1}, obtaining low bioaccumulation factors and concluding that SUC does not bioaccumulate in these organisms. Huggett and Stoddard (2011) employed 93 and 180 mg L^{-1} of SUC with species such as *Daphnia magna* and *Americamysis bahía*. Results obtained demonstrated that these concentrations of sweetener did not affect the survival, growth and reproduction of these species. In contrast, in another study with *D. magna* employed as a biomarker, it was reported for the first time that exposure to SUC at concentrations of 0.0001–5 mg L^{-1} generated alterations in physiology and mobility of these organisms. These authors attributed responses to neurological and oxidative damage (Eriksson et al., 2014). In recent times, our research team determined that SUC, at environmentally-relevant concentrations of 0.05 and 155 μg L^{-1}, are capable of inducing oxidative stress in blood, gills, liver, brain and muscle of juveniles of *C. carpio* (Saucedo-Vence et al., 2017). Data previously mentioned and specially those obtained in the group led to the proposal of the present research, due to the fact that oxidative stress has been associated with DNA alterations and apoptosis (García-Medina et al., 2017, 2013; Gómez-Oliván et al., 2017; Islas-Flores et al., 2017). Reactive oxygen species (ROS) that cause oxidative stress induced by SUC in carp are involved in the process of damage at the genetic level, not only because they are capable of interacting over DNA in a direct manner, but also because they affect the transduction of signals, cell proliferation and intercellular communication; also, DNA damage is capable of inducing the expression of the p53 proteins, generating apoptosis or cell death (Canistro et al., 2012; Chen et al., 2018; Gómez-Oliván et al., 2014; SanJuan-Reyes et al., 2015; Schieber and Chandel, 2014). The alterations to DNA were monitored through the comet assay (single-cell gel electrophoresis) [Jha, 2008], micronucleus test (Bolognesi and Cirillo, 2014; Bolognesi and Hayashi, 2011) and apoptosis through caspase-3 activity (Kurokawa and Kornbluth, 2009; Macip et al., 2003) and the TUNEL assay (Formigari et al., 2007). The aforementioned tests are very important to measure the impact by the presence of emerging contaminants (such as SUC) in water bodies. To evaluate DNA damage and apoptosis, the following biomarkers are used: 1) Comet assay or single-cell gel electrophoresis: it is a rapid and sensitive technique used in aquatic species since it detects a broad spectrum of DNA lesions, is flexible and low cost and specific endonucleases are used to recognize several types of damage in bases purine and pyrimidine of DNA. Its use has been extended to a variety of areas, including environmental monitoring and genetic ecotoxicology (de Lapuente et al., 2015; Gajski et al., 2019); 2) The micronucleus test: is a complementary test to the comet assay to detect cytoplasmic corpuscles of similar characteristics to the nucleus but of smaller size identified in the interface of the mitosis and can be originated by the inclusion in the nuclear membrane of fragments of both broken off or whole chromosomes during the karyokinesis and consequently are indicators of the action of clastogenic or aneugenic xenobiotics (Sargsyan et al., 2019; Zapata et al., 2016); 3) Caspase-3 activity: is responsible for executing the process of cell death and activating enzymes that degrade multiple proteins (such as p53, Bcl2 and Bax) with the breakdown of cellular architecture. In addition, to being important biomarkers in ecotoxicology since the process of apoptosis its expression is increased (Arslan et al., 2017); y 4) The detection of DNA fragments *in situ* using the terminal deoxyribonucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labelling (TUNEL assay); which is a complementary technique to the determination of caspase- activity 3 and is one of the most used tests in the detection and quantification of the apoptotic phenomenon (resulting in nuclear and cytoplasmic fragmentation and the formation of apoptotic bodies attached to the membrane) [Orozco-Hernández et al., 2018].

It is important to note that, in this study, *Cyprinus carpio* was used as a biomarker, due to the fact that the species is easy to culture, maintain the laboratory and is sensitive to contaminants such as SUC (Formigari et al., 2007). For all the aforementioned findings, the aim of this study was to evaluate cellular and genetic damage induced by two different environmentally-relevant of SUC concentrations (0.05 and 155 μg L^{-1}) in cell blood of the teleost *C. carpio* using DNA damage and apoptosis biomarkers.

2. Materials and methods

2.1. Test substance

The sucralose standard (1,6-dichloro-1,6-didesoxy-β-D-fructofuranosyl-4-chloro-4-desoxy-α-D-galactopyranoside) utilized in the study was purchased from Sigma-Aldrich (St. Louis, MO). The purity of the standard was ≥98%, $C_{12}H_{19}C_{l3}O_8$, with a molecular weight of 397.63 (CAS number 56038-13-2).

For the determination of SUC in water and blood, the reagents employed were HPLC-grade. The formic acid for LC-MS analysis was

purchased from Merck (Darmstadt, Germany) and nitrogen gas was obtained from INFRA, S.A. de C.V. (Mexico City, Mexico). Ultrapure water was obtained using a purification system provided by Merck Millipore. The acetonitrile was purchased from Sigma-Aldrich. The reagents employed in the DNA damage, apoptosis and oxidative stress biomarkers were acquired from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

2.2. Collection and maintenance of common carp (Cyprinus carpio)

Cyprinus carpio specimens were obtained from the carp culture facility center in Tiacaque, State of Mexico, transported in polyethylene bags with water and oxygen to Environmental Toxicology Lab in the Chemistry Faculty of the Autonomous University of the State of Mexico. Later, the organisms were maintained in 160-L-capacity fish tanks for an acclimatization time of twenty days, at room temperature, with natural light/dark (12:12 h) photoperiod, continuous oxygenation, pH 7.4 and daily feeding with Pedregal SilverTM fish food (Gómez-Oliván et al., 2017).

2.3. Experimental design to evaluate DNA damage and apoptosis

The tests were performed using specimens of 12.01 ± 0.27 cm long and weighing 28.13 ± 1.4 g. Toxicity tests consisted in the incorporation of SUC at environmentally-relevant concentrations of 0.005 μg L^{-1} and 155 μg L^{-1} (Arbeláez et al., 2015).

For this, fifteen test systems with 7 L and fifteen fish each, were employed: five of them were used for concentration 1 (c1 = 0.05 μg L^{-1}), five used for concentration 2 (c2 = 155 μg L^{-1}) and five corresponding to the control group (without SUC). Exposure times were 12, 24, 48, 72 and 96 h. The study was carried out in triplicate. In the experiment, a total of 225 organisms were employed. Exposures were static, *i.e.* no food was provided to specimens and no changes of water were made during the experiment. Later, carps were removed from the systems and placed in a fish tank containing 0.02 mg mL^{-1} of eugenol as an anesthetic. The blood samples were obtained of the tail vein with a hypodermic needle of 1 mL, previously heparinized. Blood samples were used to determine the DNA damage (comet assay and micronucleus test) and apoptosis (caspase-3 activity and TUNEL assay), using the methodologies described below:

2.4. Test to evaluate DNA damage

2.4.1. Single-cell gel electrophoresis (comet assay)

DNA damage evaluation by single-cell gel electrophoresis (*comet assay*) was carried out in five consecutive steps: 1) suspension of lymphocytes in agarose, 2) release of DNA in lysis solution, 3) unicellular electrophoresis, 4) neutralization, and 5) unicellular stain with ethidium bromide. For this purpose, the methodology established by Tice et al. (2000) was used. One day before the test, slides were prepared by placing a 100 μL layer of 1% high melting point agarose. The next day, 10 μL of the lymphocyte suspension was mixed with 75 μL (0.75%) low melting point agarose, and then 75 μL of this mixture were placed on previously prepared slides. For the DNA release, the slides were placed for 1 h at 4 °C in a copplin glass with the lysis solution (2.5 M sodium chloride, 10 mM Trizma, 10% DMSO, 1% triton and pH 10). The slides were then placed in the electrophoresis chamber under the following conditions: time 20 min, 900 mL cold alkaline solution at 4 °C (300 mM sodium hydroxide and 1 mM EDTA) at pH 13, 300 mA, 25 V and pH > 13. The process was stopped with a neutralization buffer (0.4 M of Trizma base) at a pH 7.4.

Finally, the slides were dried for 6 h, stained with 50 μL of ethidium bromide and examined under the fluorescence microscope (Zeiss) with the program Image-pro plus 5.0 (Media Cybernetics) and a wave filter of 450–490 nm. 100 measurements were made per treatment and the damage was obtained by means of the DI, which is the ratio of the length of the tail of the comet and the nuclear diameter.

2.4.2. Micronucleus test

1000 blood cells were analyzed and the content of micronucleus was determined by the method of Çavaş and Ergene-Gözükara (2005). A drop of blood from the carps (exposed to two different concentrations of environmentally relevant SUC) was placed on a slide; a smear was performed and this was stained with 10% Giemsa stain for 9 min. Micronucleus were observed as DNA fragments in the erythrocytes under an optical microscope.

The criteria used for determining the micronucleus presence (MN) were the non-linking of the small ovoid or round nucleus with the main nucleus, color and intensity of stain similar to the main cell nucleus (Kim and Hyun, 2006) and the diameter of 1/5–1/20 of the main nucleus.

2.5. Tests to evaluate apoptosis

2.5.1. Determination of caspase-3 activity

2.5.1.1. Preparation of cell extract. Positive control consisted of Jurkat cells (ATCC # TIB-152), density of 10^6 cells mL^{-1} and 50 ng mL^{-1} of anti-Fas mAb (clone # CH-11, MBL International, Cat. # SY-001). For the negative control (inhibitor caspase-3), the same cells and 125 μL of apoptosis inhibitor *Z*-DEVD-FMK [Z-Asp(OMe)-Glu(Ome)-Val-Asp(Ome)-fluorometihyketone] (irreversible inhibitor of caspase-3) were added.

Both solutions were incubated in darkness for 16 h at 37 °C in a humidified atmosphere with 5% CO_2. Cell extracts were obtained by centrifugation at 450 ×*g* at 4 °C for 10 min. Pellet was washed with PBS at 4 °C and resuspended in cell lysis buffer at a concentration of 10^8 cells mL^{-1}. The cells were lysed by freezing-thawing, and incubated on ice for 15 min. The cell lysates were centrifuged at 15,000 ×*g* at 4 °C for 20 min, finally the supernatant was the cell extract used in the test.

2.5.1.2. Colorimetric assay. The indications established in the kit were followed (CaspACETM, Promega, Madison, WI). In microplates with flat bottom were prepared the following systems:

1) Reaction blank: 32 μL of the caspase buffer [HEPES 312.5 mM, pH 7.5; sucrose 31.25%; CHAPS 0.3125% (3 - [(3-Colamidopropyl) -dimethylammonium] -propane sulfonate)]; 2 μL DMSO; 10 μL dithiothreitol (DTT, 100 mM) and 54 μL of deionized water;

2) Exposed group: 32 μL of caspase buffer; 2 μL of DMSO; 10 μL of DTT, 20 μL of blood and 54 μL of deionized water;

3) Positive and negative control: 32 μL of the caspase buffer; 2 μL of DMSO; 10 μL of DTT; 20 μL of the cell extract (for each case) and 34 μL of deionized water.

To each system, 2 μL of the substrate DEVD-pNA was added. The microplates were covered with parafilm and incubated at 37 °C for 4 h. Finally, the absorbance at 405 nm was read and the specific activity of caspase-3 calculated. The results were expressed as nM of free pNA h^{-1} μg $protein^{-1}$.

2.5.2. TUNEL assay

The ApopTag Fluorescein S7110 kit (Chemicon, Temecula CA) was used to identify apoptotic cells. The kit consists of 6 sequential steps that consisted of: 1) obtaining lymphocytes, 2) cell preservation, 3) cellular fixation, 4) hydration, 5) cell labeling and 6) cell staining. For the first step, a sample of 300 μL of blood plasma (from carps exposed to environmentally relevant concentrations of AMX) was placed in an eppendorf tube and centrifuged at 3000 ×*g* for 5 min at 4 °C to obtain lymphocytes. These cells were resupended with 50 μL of mounting solution. A 1 μL of this solution was placed on a slide previously prepared with poly-L-lysine and dried for 5 min at 60 °C. Once the cellular material was dried, it was fixed with cold acetone for 10 min (step 2). For the third step, the slides were placed in serial solutions of xylene-absolute

alcohol, at 96, 80, 70, 60, 50% (*v*/v) and finally 30s in distilled water. Hydrated cells were added with 1 μL of proteinase k (20 μg mL^{-1}) for 10 min. Three washes were made with PBS (0.138 M NaCl, 0.0027 M KCl) pH 7.4 for 1 min and 60 μL of the equilibrium buffer was added, followed by an incubation at 37 °C for 1 h, previously adding 65 μL of the TdT enzyme (step 4). For cell labeling (step 5) a wash with PBS was performed and the anti-FITC conjugate was added at room temperature for 30 min. Finally, for cell staining (step 6), propidium iodide (1.5 μg mL^{-1}) was used as a dye and examined in an epifluorescence microscope with a digital camera.

The negative control consisted of cells treated as described above, but without the addition of TdT, and the positive control consisted of cells treated with DNase I (1 μg mL^{-1}). A total of 100 cells were analyzed per fish and apoptosis was expressed as the percentage of TUNEL positive cells in 100 cells.

2.6. Oxidative stress determination

In order to corroborate that oxidative stress was the mechanism through which the SUC generated DNA alterations and apoptosis, an additional experiment was performed with systems similar to those mentioned in Section 2.3 (using the same conditions, exposure times and a control of each exposure time). Once each exposure time had been finalized, the organisms of each system were placed in a tank of eugenol (0.02 mg mL^{-1}), with the finality of anesthetizing and obtaining the blood samples of the tail vein with a hypodermic needle. Six hundred microliters of blood samples were collected in heparinized tubes, placed in 1 mL of Tris buffer solution pH 7. The supernatant was centrifuged at 12, 500 ×*g* for 15 min at −4 °C. The serum obtained was used to determine the oxidative stress determinations: hydroperoxide content (HPC) [Jiang et al., 1992], lipidperoxidation level (LPX) [Buege and Aust, 1978], protein carbonyl content (PCC) [Levine et al., 1994 modified by Burcham, 2007] and superoxide dismutase activity (SOD [Misra and Fridovich, 1972] and catalase activity (Radi et al., 1991).

2.7. Determination of SUC in water and plasma of common carp by liquid chromatography-tandem mass spectrometry (HPLC-MS/MS)

In order to determine the concentration of SUC in water and plasma of *Cyprinus carpio*, the methodology established by Saucedo-Vence et al. (2017) was used, for which a stock solution of sweetener (1000 μg L^{-1}), using methanol and ultrapure water (50%/50%). This solution was stored at −20 °C in bottles covered from the light. For the determination of this compound, an Agilent 1290 Infinity HPLC system coupled to an Agilent 6430 Triple Quadrupole MS system capable of ionization by electrospray (ESI). The injection volume was 50 μL. The chromatographic conditions were: the process of separation was carried out with an elution gradient with acidulated ultrapure water with formic acid at pH 2.5 (solvent A) and acetonitrile (solvent B) as a mobile phase. The gradient started isocratically at 5% of B for 3 min and later incremented to 75% at 6 min. Afterwards, it increased at 100% at 1 min, remained constant for 1 min and finally returned to 5% of B at 1 min. Eclipse Plus C18 columns (2.1 × 50 mm, 1.8 μm) were employed. The temperature was of 25 °C and the flow was of 0.4 mL min^{-1}. The elution period of SUC was <8 min. The analysis was carried out in negative ionization mode employing Multiple Reaction Monitoring (MRM) using the transitions of the ion precursor/product 395/359.

The ESI-MS conditions employed were: nebulizer pressure of 45 psi, drying gas flow (N_2) of 11 L min^{-1}, drying gas temperature of 350 °C and capillary voltage of 4000 V.

For the determination of SUC in water samples, the systems used in Section 2.3 were employed, and of each system, 10-mL samples were taken. The samples were acidified with 1 M HCl and SUC was extracted from 1-mL water samples with 5 mL of methanol:water (1:1). The mixture was centrifuged at 1800 ×*g* for 10 min, and the organic layer obtained was used for its analysis. For the plasma samples (the samples obtained in Section 2.3), cold acetone was added and was centrifuged at 2500 ×*g* for 5 min. The supernatant was separated and SUC concentration was determined.

2.8. Correlation between the SUC plasma concentrations in C. carpio and the biomarkers of DNA damage and apoptosis

Using the Pearson test, the correlation between SUC plasma levels and DNA damage biomarkers (single-cell gel electrophoresis and micronucleus test) and apoptosis biomarkers (caspase-3 activity and TUNEL assay) was established, using the Sigmastat v2.03 program.

2.9. Statistical analysis

The normality of the data was established and the homoscedasticity through the Shapiro-Wilk and Levene test, respectively. Posteriorly, a *Bonferroni post hoc* test was used to evaluate significative differences. The Sigmastat v2.03 program was employed.

Oxidative stress biomarkers results were evaluated by one-way analysis of variance (ANOVA), followed by a multiple comparison test (*Bonferroni*) with $p\text{-value} < 0.05$. The Sigmastat v2.03 program was employed.

3. Results and discussion

3.1. DNA damage

3.1.1. Single-cell gel electrophoresis (Comet assay)

A tendency towards increased DNA damage was observed from 24 h and up to 72 h (Fig. 1). In both concentrations, a maximum increase of biomarker at 72 h was observed. In c1 (0.05 μg L^{-1}), there was a significant increase at 24, 48 and 72 h ($p < 0.05$), compared to the control group. In c2 (155 μg L^{-1}), significant increases were observed at 48 and 72 h respect to control group ($p < 0.05$).

After comparing exposure times, it can be observed that in c1 at 72 h, significant decreases were observed respect to 12, 24, 48 and 96 h. In the case of c2, statistically-significant decreases were observed at 12, 24 and 96 h compared to 72 h.

The comet assay is a test which detects damage produced by a single or double break in DNA, oxidative damage induced and cross-linking of DNA-DNA/DNA-protein, is rapid a sensitive method (de Lapuente et al., 2015). This test can be applied to any nucleated eukaryotic cell type which can be obtained as single cells or nuclear suspensions (Bolognesi et al., 2019). The results evidenced by the comet assay are shown in Fig. 1 and demonstrated that the two tested concentrations in this study generated DNA alterations. The highest expression of this biomarker was evidenced at 72 h. These results could be due to the oxidation of bases [for example, 7,8-dihydrodeoxyguanosine (8-oxoG) and 8-oxoguanine] and for the generation of interruptions in the DNA chain by the reactive oxygen species (ROS). As was previously established by our research team, SUC at concentrations of 0.05 and 155 μg L^{-1} is capable of inducing oxidative stress in blood, liver, brain, gills and muscle of *C. carpio* (Saucedo-Vence et al., 2017). The results of this investigation are consistent with those obtained by Eriksson et al. (2014), which determined that SUC, at concentrations of 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000 and 5000 μg L^{-1}, generated oxidative damage, evidenced by significant increases in the levels of TBARS in *Daphnia magna*, as well as an increase in the concentrations of acetylcholinesterase, provoking neurotoxicity in the daphnid. The same research team, in 2012, demonstrated that concentrations of 0.5, 5 and 500 μg L^{-1} of SUC affected the physiology and locomotion of *D. magna* and *G. zaddach* (Wiklund et al., 2012).

It is well-known that ROS, as well as the radical anion superoxide (O_2^*) and the radical hydroxyl ($^*OH$) have the capacity of extracting protons or attacking molecules in sites of high electron density, generating secondary radical species which were capable of suffering

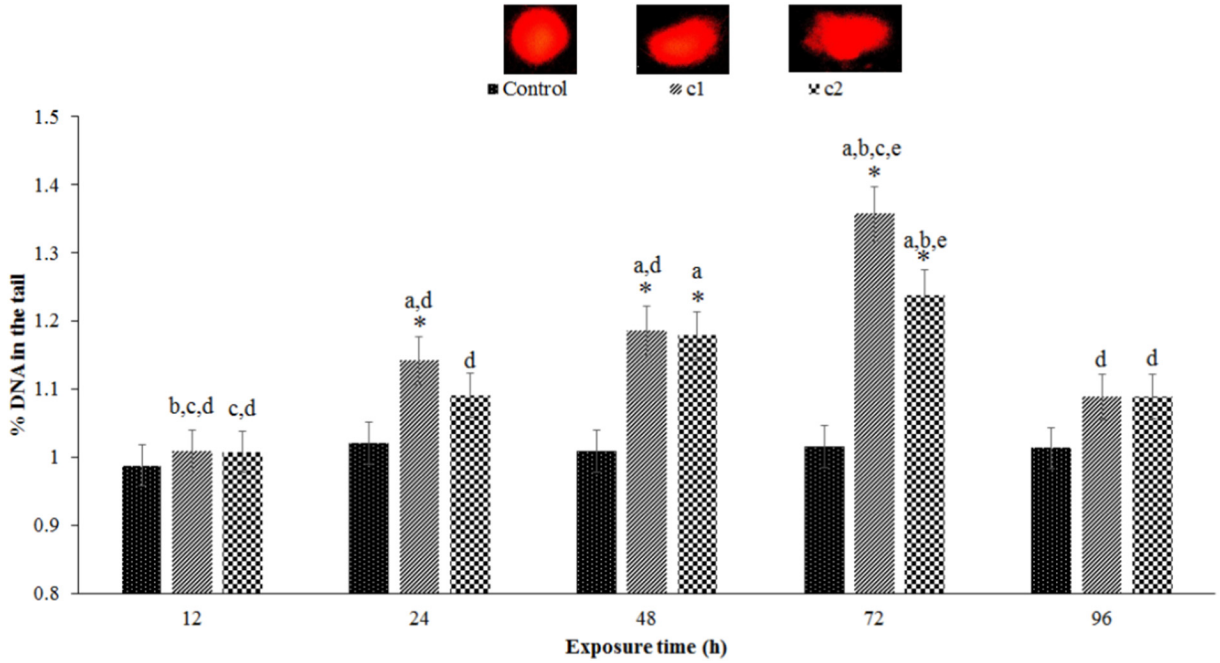

Fig. 1. Determination of DNA damage through the comet assay in blood cells of *Cyprinus carpio* exposed to environmentally-relevant concentrations of SUC (0.05 and 155 μg L^{-1}) at different exposure times. The bars represented the mean ± SEM of the damage index values of five specimens by concentration and by exposure time. The assay was carried out in triplicate. Significantly different from: *control group; [a]12 h; [b]24 h; [c]48 h; [d]72 h; [e]96 h (*Bonferroni post hoc, $p < 0.05$*).

intramolecular rearranging. In nucleic acids such as DNA, puric and pyrimidine bases, as well as sugar, are susceptible to ROS attacks, which lead to modifications in the bases and ruptures in DNA chain (Valavanidis et al., 2006; Zaremba and Oliński, 2010). Specially, the radical hydroxyl *OH has the capacity of hydroxylating C5 and C6 of the pyrimidine bases and C4, C5 and C8 of the purine bases in the DNA molecules (Evans et al., 2004; Gruber et al., 2018). The prior findings explain the results observed in the comet assay; however, after 72 h, a decrease in the biomarker was observed at 96 h, which would demonstrate that, at this time, the reparation mechanisms in carp were expressed (Sassa et al., 2014).

The repair processes of each species and cell type are different. (Mohanty et al., 2011) studied DNA damage and repair kinetics in *Labeo rohita* hepatocytes by exposure to phorate, organophosphorus pesticide, and their results showed that decreases in DNA damage were observed after 72 h of exposure. In another study, (Saleha Banu et al., 2001), showed that in *Tilapia mossambica,* exposed to different concentrations of monocrotophos, a gradual decrease in DNA damage was observed after 48 h, reaching a total recovery at 96 h. Another study conducted by (Jin et al., 2004), showed that in carps exposed to different concentrations of *N-nitro-N-nitrosoguanidine* and *benzo[α] pyrene* for 7 days, the damage to DNA in hepatocytes *Cyprinus carpio*

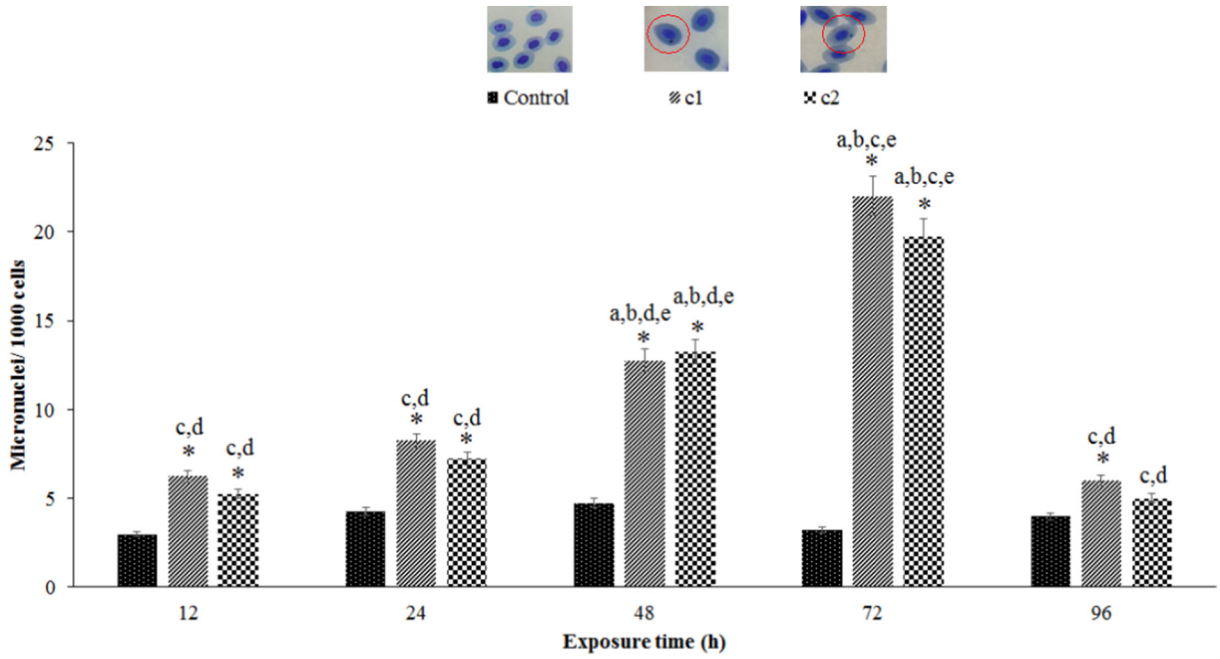

Fig. 2. Frequency of micronuclei in blood cells of *Cyprinus carpio* exposed to environmentally-relevant concentrations of SUC (0.05 and 155 μg L^{-1}) at different exposure times. The values are the mean of three repetitions ± SEM. Significantly different from: *control group; [a]12 h; [b]24 h; [c]48 h; [d]72 h; [e]96 h (*Bonferroni post hoc, $p < 0.05$*).

was increased in time and concentration dependent, observing a gradual repair of DNA damage at 48 h. This suggests that DNA repair systems are dependent on the threshold of xenobiotic, which would lead to the DNA repair enzymes being activated depending on how much xenobiotic accumulates in the cells above a threshold (Ching et al., 2001), as well as, depending on each species, cell and xenobiotic type.

3.1.2. Micronucleus test in C. carpio blood cells exposed to SUC

In Fig. 2, increases in the presence of MN from 24 h and up to 72 h, with respect to control group were observed in c1 y c2. In c1, significant increases at 12, 24, 48, 72 and 96 h were observed respect to control group, respectively. In c2, increases at 12, 24, 48 and 72 h were observed compared to control group, respectively ($p < 0.05$). Comparing exposure times for c1, significant decreases were observed at 12, 24, 48 and 96 h respect to 72 h ($p < 0.05$). In c2, a similar behavior was observed, at 12, 24, 48 and 96 h respect to 72 h ($p < 0.05$).

In the evaluation of genotoxicity, it was also observed that the presence of micronucleus presented the highest response in both concentrations of SUC at 72 h, and posteriorly at 96 h; we observed the same behavior that was found in the comet assay. The presence of micronucleus is a genetic damage biomarker which has been widely employed in ecotoxicology (de Lima Cardoso et al., 2018). The micronucleus (MN) form due to the fact that during the process of cell division, the DNA contained in the nucleus replicates and equally divides, giving way to twin daughter cells; when this process is produced in an erroneous form due to errors during the process of replication and its posterior division, to chromosomic ruptures or to genotoxic substances (SUC), the genetic material that separated is not incorporated into the nucleus of the daughter cells and give origin to nucleus of a smaller size which are denominated MN (Hussain et al., 2017). The results obtained in this biomarker can be specifically due to the DNA breakdown, which had been demonstrated in this study with the results of the comet assay or the lack of segregation of chromosomes which result in aneugenic and clastogenic effects caused by ROS. The specific mechanism through which genotoxic effects are being generated is through oxidative stress that is discussed later in this study. As in the comet assay, a repair process at 96 h was observed in this biomarker.

Both in the comet assay and in the micronucleus test we observed a very specific phenomenon, and it was that at the SUC concentration of 0.05 μg L^{-1} observed a greater or similar response with respect to the SUC concentration of 155 μg L^{-1} (3100 times higher). Currently, it is already well documented for various xenobiotics that low concentrations of these compounds can regulate the biological/physiological adaptive functions resulting in non-linear dose/response relationships, presenting positive and negative responses at low and high concentrations, respectively (hormesis phenomenon) [Agathokleous et al., 2018; Calabrese, 2017; Hashmi et al., 2014]. Emerging contaminants such as SUC can generate these hormetic responses in different organisms (McGinnis et al., 2019).

3.2. Apoptosis in blood cells of C. carpio exposed to SUC

3.2.1. Caspase-3 activity in blood cells of C. carpio exposed to SUC

As shown in Fig. 3, in c1 significant increases ($p < 0.05$) with respect to the control group were observed at 24, 48, 72 y 96 h in the specific activity of caspase- 3. In c2, significant increases at 12, 24, 72 and 96 h were observed with respect to control group ($p < 0.05$). After comparing the positive control with c1, statistically-significant decreases were observed at 12, 24, 48 and 72 h ($p < 0.05$), and at 96 h a significant increase was observed ($p < 0.05$). When the positive control was compared respect to c2 at 12 and 48 h, a decrease was observed ($p < 0.05$). In contrast to 24 and 96 h, the biomarker increased significantly ($p < 0.05$). Comparing the negative control (inhibitor caspase-3) with c1 and c2, a significant difference is observed at all exposure times ($p < 0.05$).

The damage to DNA induced by ROS could generate mutagenicity or cytotoxicity, depending if DNA and/or RNA polymerases are blocked or not. The biological consequences of the DNA alterations could range from induction of programmed cell death (apoptosis) to necrosis (Maynard et al., 2008).

In the present work, it was demonstrated the SUC was also capable of inducing apoptosis. This was shown through the biomarkers of caspase-3 activity and the TUNEL assay. In both biomarkers, significant increases with regard to the control group in both of the concentrations employed, was observed (Figs. 3 and 4), with a maximum response at 96 h. Caspase-3 is an important biomarker due to the fact that it is an apoptosis executor enzyme, which is responsible for dismantling cellular architecture or cell death. Also, the intrinsic or mitochondrial route and the extrinsic route, which implicate the death receptors such as

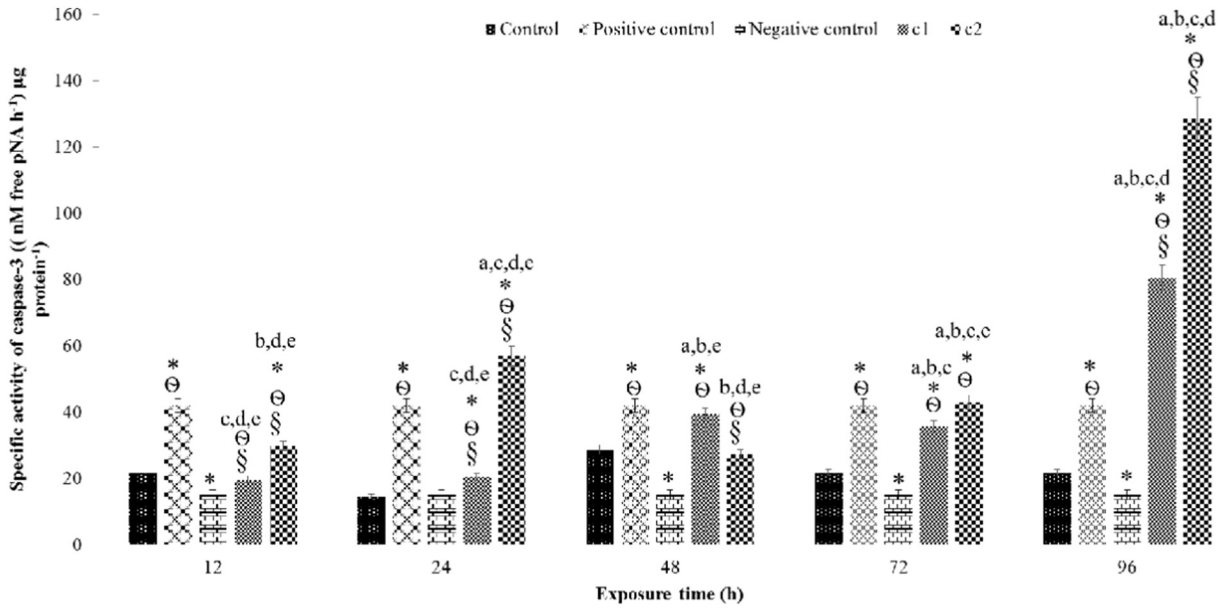

Fig. 3. Activity of caspase-3 in blood cells of *Cyprinus carpio* exposed to environmentally-relevant concentrations of SUC (0.05 and 155 μg L^{-1}) at different exposure times. The assay was carried out in triplicate. Significantly different from: *control group; §Positive control ΘNegative control (irreversible inhibitor of caspase-3) a12 h; b24 h; c48 h; d72 h; e96 h (*Bonferroni post hoc*, $p < 0.05$).

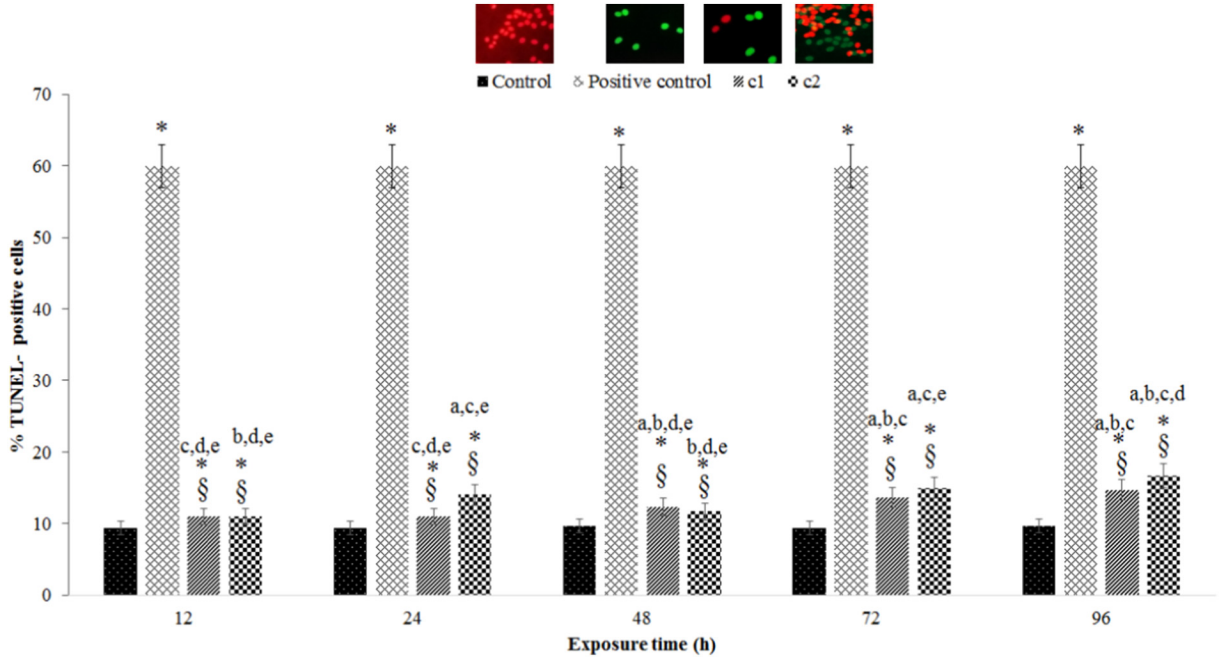

Fig. 4. Percentage of TUNEL-positive cells in blood cells of *Cyprinus carpio* exposed to environmentally-relevant concentrations of SUC (0.05 and 155 μg L^{-1}) at different exposure times. The assay was carried out in triplicate. Significantly different from: *control group; §Positive control [a]12 h; [b]24 h; [c]48 h; [d]72 h; 96 h (*Bonferroni post hoc*, $p < 0.05$).

receptor 1 of TNF (TNFR-1) and Fas (CD95), converge at the level of caspase-3 activation in order to produce apoptosis (Wang et al., 2019; Zhou et al., 2019). Until now, an exhaustive revision of international literature has not shown that SUC is involved in the induction of apoptosis in mammals and aquatic life; however, the presence of ROS and DNA damage have been associated with the activation of caspase-3 in aquatic organisms (González-Pleiter et al., 2017; Selvaraj et al., 2013).

3.2.2. TUNEL assay in blood cells of C. carpio exposed to SUC

Fig. 4 shows the results of the positive apoptotic cells found in the TUNEL assay. As can be seen, in c1, there were significant increases at 12, 24, 48, 72 and 96 h respect to control group ($p < 0.05$). In c2, the same tendency was observed ($p < 0.05$). Comparing the positive control with c1 and c2, there were significant decreases at 12, 24, 48, 72 and 96 h ($p < 0.05$).

The induction of apoptosis by exposure to SUC was corroborated by the TUNEL assay (Terminal Transferase dUTP Nick End Labeling), which was a technique for detecting the fragmentation of DNA, labeling the terminal fragment of the nucleic acids and also a confirmatory test of apoptosis (Chen et al., 2018; Zeng et al., 2014). Likewise, the TUNEL assay is very efficient in identifying DNA damage *in situ*, and to identify in blood cells, the initial stages of apoptosis and the apoptotic bodies due to exposure to xenobiotic substances (Quan et al., 2019). The findings found in both biomarkers of apoptosis evaluated in this study, could be related to oxidative stress generated in *C. carpio* by exposure to sucralose.

At low concentrations, it is considered that ROS play a fundamental role in the regulation of normal physiological functions involved in the development, progression and proliferation of the cell cycle, differentiation, migration and cell death (Bae et al., 2011; Zhang et al., 2016). In addition, ROS have been related to the activation of various cell signaling pathways and transcription factors, such asphosphoinositide 3-kinase (PI3K)/Akt, mitogen-activated protein kinases (MAPK), nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/Kelch like-ECH-associated protein 1 (Keap1), nuclear factor-κB (NF-κB) and the tumour suppressor p53, responsible for mediating cell survival or cell death processes such as autophagy and apoptosis (Dickinson and Chang, 2011; Kaminskyy and Zhivotovsky, 2014).

3.3. Oxidative stress results

3.3.1. Cell oxidation biomarkers

Fig. 5 shows the results of the cell oxidation biomarkers: **5A** shows the content of hydroperoxides determined by exposure to SUC, as can be seen, in the c1 significant increments were observed with respect to the control group at 12, 24, 48 and 72 h, and in c2 at 24, 48 and 72 h ($p < 0.05$). **5B** showed the MDA content due to exposure to SUC, for c1 increments were observed at 12, 24, 48 and 72 h, and for c2 at 48 and 72 h, compared to the control group ($p < 0.05$). Finally in **5C**, increments were observed at 48 and 72 h for c1 and at 12, 24, 48 and 72 h for c2 respect to the control group ($p < 0.05$), in the content of carbonylated proteins.

3.3.2. Antioxidation biomarkers

Fig. 6 shows the antioxidation biomarkers. In **6A** the activity of the SOD is shown, for both c1 and c2, significant increments were observed at 12, 24, 48 and 72 h compared to the control group ($p < 0.05$). In **6B** the activity of catalase was observed by exposure to SUC. In c1, significant increments were observed at 48, 72 and 96 h with respect to the control group ($p < 0.05$), and in c2 there was an increase in all the exposure times with respect to the control ($p < 0.05$). However, at 72 h, the maximum increase was observed.

As previously explained, the increase in biomarkers of DNA damage and apoptosis due to exposure to SUC were attributed to the presence of ROS, which also causes oxidative stress in blood cell of *C. carpio*. In a study previously carried out by our research group, we had already established that the SUC is capable of inducing oxidative stress in blood, liver, gill and brain cells of the carp. In order to corroborate these findings and to strengthen the results obtained in this study, we performed biomarkers of cellular oxidation and antioxidation at the two environmentally relevant concentrations of SUC in the blood cells of *Cyprinus carpio*. The results confirmed that the SUC is able to induce an increase in the content of hydroperoxides, lipid peroxidation level and content of carbonylated proteins and to generate an activation of the enzymes superoxide dismutase and catalase. The most pronounced effects in the biomarkers of cellular oxidation and antioxidation were found in c2 and also at 72 h, with recovery at 96 h. These findings are

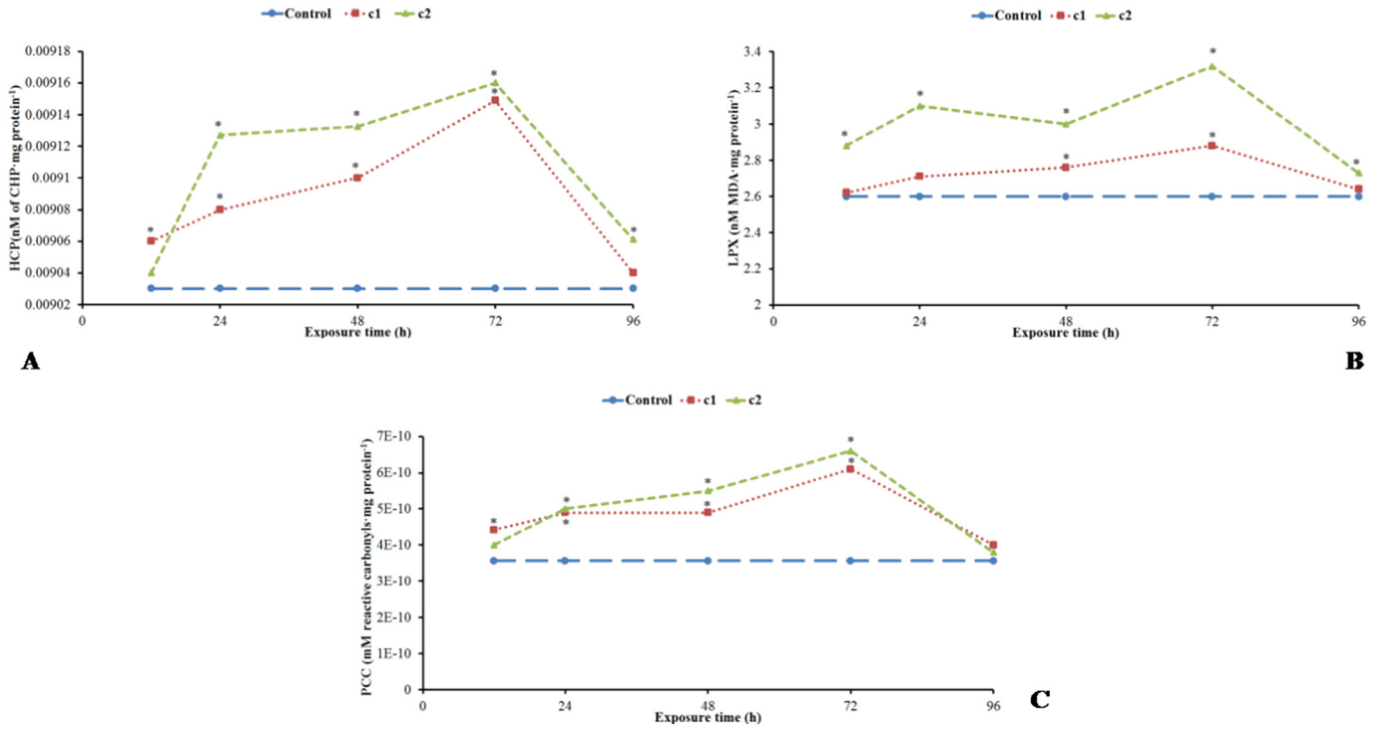

Fig. 5. Cell oxidation biomarkers in blood cells of *Cyprinus carpio* exposed to environmentally-relevant concentrations of SUC (0.05 and 155 μg L^{-1}) at different exposure times. The assay was carried out in triplicate. **5A** = Hydroperoxide content, **5B** = Lipid peroxidation level and **5C** = Protein carbonyl content. Significantly different from: *control group (*Bonferroni test, $p < 0.05$*).

consistent with those obtained by (Eriksson et al., 2014), who determined that due to SUC exposure, neurological and oxidative damage was observed in *Daphnia magna*, generating important consequences in the behavior and physiology of cladoceran.

3.4. Determination of the concentration of SUC in water and plasma of *Cyprinus carpio*

Table 1 shows the data of SUC concentrations in water and plasma of *Cyprinus carpio* at different exposure times. As can be seen, the plasma concentrations of SUC increment to 9% in c1 and 4.9% in c2. In water, a tendency to decrease in the concentration of SUC with respect to time was observed; for c1, the decrease was 60% and for c2 36.8%, respect to the initial quantity of the systems at 96 h.

Although SUC has demonstrated that it does not biotransform in human beings, it has demonstrated having a high stability and being resistant to the environment (Grice and Goldsmith, 2000), which favors its bioconcentration in blood, as was demonstrated in this study since, with the passing of time, the concentration of SUC decreased within the exposure systems, but increased its concentration in the blood of the carp. These data show that there is a direct correlation, since increasing concentration of SUC in blood increases DNA damage and apoptosis biomarkers.

In the previous study done by our research group, we observed that the bioconcentration factors were lower than 1, which implies that the sweetener is not able to bioconcentrate (Saucedo-Vence et al., 2017). However, in this study we work with another type of cells. We consider blood as a system where toxins arrive after being absorbed to distribute throughout the body and produce its toxic effects. In addition, the time in which we conducted the trials was short. For this reason there was accumulation of the SUC in plasma. Another reason why the SUC could accumulate in the blood is due to its high stability and resistance

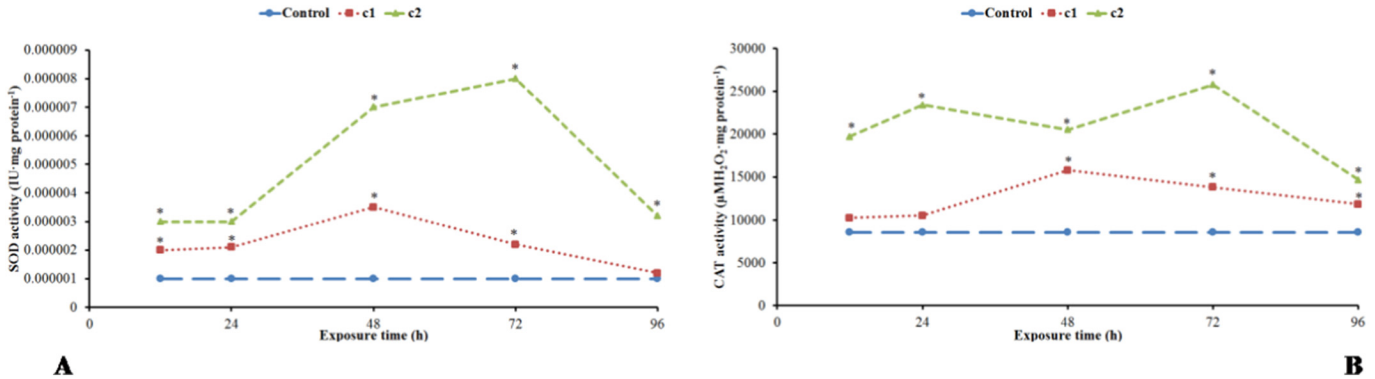

Fig. 6. Antioxidation biomarkers in blood cells of *Cyprinus carpio* exposed to environmentally-relevant concentrations of SUC (0.05 and 155 μg L^{-1}) at different exposure times. The assay was carried out in triplicate. **6A** = Superoxide dismutase activity, **6B** = Catalase activity. Significantly different from: *control group (*Bonferroni test, $p < 0.05$*).

Table 1
Concentrations of SUC in the water and plasma of common carp *C. carpio* at different exposure times.

Exposure concentration	Exposure time	SUC in water system ng L^{-1}	SUC in blood carp *Cyprinus carpio* ng L^{-1}
Control group	12	ND	ND
	24	ND	ND
	48	ND	ND
	72	ND	ND
	96	ND	Nd
c1	12	0.04 ± 0.005	$1 \times 10^{-3} \pm 1 \times 10^{-4}$
0.050 μg L^{-1}	24	0.04 ± 0.003	$1 \times 10^{-3} \pm 1 \times 10^{-4}$
	48	0.03 ± 0.004	$2.5 \times 10^{-3} \pm 2 \times 10^{-4}$
	72	0.03 ± 0.001	$4.2 \times 10^{-3} \pm 3 \times 10^{-4}$
	96	0.02 ± 0.003	$4.5 \times 10^{-3} \pm 1 \times 10^{-4}$
c2	12	131.9 ± 2.7	5.8 ± 0.6
155 μg L^{-1}	24	128.4 ± 3.1	6.1 ± 0.9
	48	121.4 ± 3.9	6.5 ± 0.5
	72	112.6 ± 4.1	7.6 ± 0.8
	96	97.9 ± 2.6	8.3 ± 0.7

to hydrolysis (Tollefsen et al., 2012). However, to verify the bioconcentration capacity of the SUC, we would have to expose it for longer times.

3.5. Correlation of the DNA damage and apoptosis biomarkers with the plasma levels of SUC in common carp C. carpio

The correlation between SUC plasma levels and DNA damage and apoptosis biomarkers are shown in Table 2. As can be seen, for both concentrations, the correlations were similar; however, for the comet assay and the micronucleus test, the highest effects were observed at 72 h. For apoptosis biomarkers such as caspase-3 activity and the TUNEL assay, the highest reponses were observed at 96 h.

The data obtained in this study allow to clarify, more precisely, the genotoxic, apoptotic and oxidative damage due to exposure to SUC in *Cyprinus carpio*, which is a species of economic interest. However, it is necessary to carry out more studies involving the use of different molecular biomarkers (for example, embryotoxicity and teratogenicity) in order to explain the toxicity profile of this substance, as well as to determine the risk due to the presence of this substance in aquatic ecosystems.

Table 2
Pearson's correlation between plasmatic SUC concentrations and DNA damage and apoptosis biomarkers in *C. carpio*. Correlation coefficients >0.5 are significant (shown in bold).

Biomarkers	Time (h)	SUC	
		0.05 μg L^{-1}	155 0.05 μg L^{-1}
Comet assay	12	−0.181	**−0.812**
	24	**−0.899**	0.198
	48	−0.111	**−0.755**
	72	**−0.923**	**0.545**
	96	0.081	**−0.589**
Micronucleus test	12	**−0.792**	**1.000**
	24	**−0.960**	**−0.981**
	48	**1.000**	**−0.924**
	72	**0.519**	**0.866**
	96	**0.866**	0.277
Caspase-3 activity	12	**−0.990**	**−0.925**
	24	**0.501**	**1.000**
	48	**−0.812**	**1.000**
	72	**−0.501**	**1.000**
	96	**−0.501**	**−0.826**
TUNEL assay	12	**−0.501**	**0.501**
	24	**0.501**	−0.188
	48	0.327	−0.263
	72	−0.397	**−0.501**
	96	0.240	0.090

4. Conclusions

The results obtained in this study allow us to conclude that SUC, at environmentally-relevant concentrations of 0.05 and 155 μg L^{-1}, was capable of inducing alterations to DNA, generate apoptosis and oxidative damage in blood cells of *Cyprinus carpio*. The comet assay, MN test, caspase-3 activity, TUNEL assay and oxidative stress biomarkers demonstrated to be sensitive, dependable and fast bioassays of the exposure of this sweetener in common carp.

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