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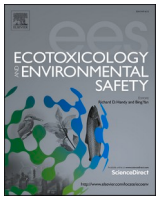

Research paper

Effects of environmental cocaine concentrations on COX and caspase-3 activity, GRP-78, ALT, CRP and blood glucose levels in the liver and kidney of the European eel (*Anguilla anguilla*)

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ABSTRACT

Cocaine is one of the most widely used illicit drugs in the world, and as a result of incomplete removal by sewage treatment plants it is found in surface waters, where it represents a new potential risk for aquatic organisms. In this study we evaluated the influence of environmental concentrations of cocaine on the liver and the kidney of the European eel (*Anguilla anguilla*). The eels were exposed to 20 ng L^{-1} of cocaine for fifty days, after which, three and ten days after the interruption of cocaine exposure their livers and kidneys were compared to controls. The general morphology of the two organs was evaluated, as well as the following parameters: cytochrome oxidase (COX) and caspase-3 activities, as markers of oxidative metabolism and apoptosis activation, respectively; glucose-regulated protein (GRP)78 levels, as a marker of endoplasmic reticulum (ER)-stress; blood glucose level, as stress marker; serum levels of alanine aminotransferase (ALT), as a marker of liver injury and serum levels of C-reactive protein (CRP), as a marker of the inflammatory process. The liver showed morphologic alterations such as necrotic areas, karyolysis and pyknotic nuclei, while the kidneys had dilated glomeruli and the renal tubules showed pyknotic nuclei and karyolysis. In the kidney, the alterations persisted after the interruption of cocaine exposure. In the liver, COX and caspase-3 activities increased (COX: $P = 0.01$; caspase-3: $P = 0.032$); ten days after the interruption of cocaine exposure, COX activity returned to control levels ($P = 0.06$) whereas caspase-3 activity decreased further ($P = 0.012$); GRP78 expression increased only in post-exposure recovery specimens (three days: $P = 0.007$ and ten days: $P = 0.008$ after the interruption of cocaine exposure, respectively). In the kidney, COX and caspase-3 activities increased (COX: $P = 0.02$; caspase-3: $P = 0.019$); after the interruption of cocaine exposure, COX activity remained high (three days: $P = 0.02$ and ten days: $P = 0.029$ after the interruption of cocaine exposure, respectively) whereas caspase-3 activity returned to control values (three days: $P = 0.69$ and ten days: $P = 0.67$ after the interruption of cocaine exposure, respectively). Blood glucose and serum ALT and CRP levels increased (blood glucose: $P = 0.01$; ALT: $P = 0.001$; CRP: 0.015) and remained high also ten days after the interruption of cocaine exposure (blood glucose: $P = 0.009$; ALT: $P = 0.0031$; CRP: 0.036). These results suggest that environmental cocaine concentrations adversely affected liver and kidney of this species.

1. Introduction

According to the World Drug Report (2019), the illicit production of cocaine worldwide reached an all-time high of 1976 tons in 2017, when it was estimated that there were 18.1 million users, with the highest rates reported in North America and Oceania, followed by Western and Central Europe and some South African countries. The consequence of this enormous use of cocaine is its presence in the surface waters of rivers and oceans worldwide (Pal et al., 2013; Seabra-Pereira et al., 2016). Indeed, after the self-administration, cocaine undergoes a

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metabolism process producing different metabolites; however, a small percentage (1–9%) remains as unchanged drug, which, together with its metabolites, is mainly excreted with urines. Thus, cocaine is found in sewage effluents and can arrive in surface waters, as the consequence of a variable ability of the sewage treatment plants of removing chemicals. Though cocaine is found in rivers and oceans at low concentrations, between 0.4 and 44 ng L^{-1} (Pal et al., 2013), its presence is a cause for concern for the health of the aquatic fauna living in contaminated waters. At environmental concentrations, cocaine induced cytotoxic and genotoxic effects in zebrafish embryos (Parolini et al., 2017); moreover, the impairment of skeletal muscle development (Monaco et al., 2016) and the accumulation of cocaine in the eyes (Niklaus et al., 2017) were observed in zebrafish larvae. Our previous studies showed that chronic exposure to environmentally relevant concentrations of cocaine induced the accumulation of this drug in tissues of the European eel (Capaldo et al., 2012) as well as changes in the endocrine system (Gay et al., 2013), skin, intestine (Gay et al., 2016), skeletal muscle (Capaldo et al., 2018) and gills (Capaldo et al., 2019). In this study, we describe the effects of environmental cocaine concentrations on the liver and kidney of the European eel. Indeed, the ability of the eels to accumulate many lipophilic pollutants and their wide diffusion in fresh and marine waters makes them excellent bio-indicators of environmental contamination and models of drug response. The following parameters were analyzed: general organ morphology; cytochrome oxidase (COX) activity, as a marker of oxidative metabolism (Lee and Hüttemann, 2014); caspase-3 activity, as a marker of apoptosis activation, since caspase-3 is the major player in the apoptotic pathway (Brentnall et al., 2013); glucose-regulated protein (GRP)78 expression, because of its role as a regulator of apoptosis and as a marker of endoplasmic reticulum (ER)-stress (Hotamisligil, 2010); blood glucose, as a stress marker in fish (Islam et al., 2020); serum levels of alanine aminotransferase (ALT), a well established biomarker of liver injury (Banaee, 2013); serum levels of C-reactive protein (CRP), as a marker of the inflammatory process (Liu et al., 2004). Moreover, the recovery ability of the eels was evaluated exposing some of the treated eels, at the end of the exposure period, to tap water only, for three and ten days, respectively.

2. Materials and methods

2.1. Chemicals

Ethyl 3-aminobenzoate, methanesulfonic acid salt 98% (MS-222) and cocaine free-base were purchased from Aldrich Chemical Corporation Inc. (Milwaukee, WI, USA) and Sigma-Aldrich Inc. (St. Louis, MO, USA), respectively.

2.2. Animals

The experiment was performed on adult male European eels (*Anguilla anguilla*), silver stage, obtained from a local fish dealer. Before starting the experiment, the eels (39.66 ± 0.41 cm; 86.25 ± 1.58 g; mean ± s.d.) were allowed to acclimatize to the laboratory conditions for a month in 300 L glass aquaria and under the natural photoperiod. The parameters of the water, that was dechlorinated and well-aerated, were as follows: temperature 15 °C ± 1 °C; pH 7.4 ± 0.8, salinity 0, dissolved oxygen 8.0 ± 0.7 mg L^{-1}; ammonia <0.1 mg L^{-1}; mean ± s.d., as previously described (Capaldo et al., 2019). The water was renewed every 24 h. Since at the silver stage the eels fast, they were not fed. The experiment complied with the EU Directive 2010/63/EU for animal experiments and was authorized by the General Direction of Animal Health and Veterinary Drugs of the Italian Ministry of Health. Throughout the experiment, the institutional guidelines for care and use of laboratory animals were respected.

2.3. Experimental design

After acclimatization, the eels were randomly assigned to one of five groups; untreated control, vehicle control, cocaine exposed, post-exposure recovery three days and post-exposure recovery ten days, each group containing ten specimens. Since the experiment was performed in triplicate, for each experimental group, three 300-L glass aquaria, each containing ten specimens, were set up (30 specimens per experimental group). The experimental conditions were as described above; in each aquarium the water was renewed every 24 h, before the administration of cocaine. Considering that in surface waters cocaine was found in concentrations between 0.4 and 44 ng L^{-1} (Pal et al., 2013; Li et al., 2016), we exposed the eels to a nominal dose of 20 ng L^{-1}. Every 24 h, three groups of eels received 1 mL of a stock solution (0.006 mg mL^{-1} of cocaine free-base in ethanol), which was added to the aquaria after the water changes; the percentage ethanol concentration in the water was 0.000333%. The water removed from the aquaria was stored in special containers for three days before being discharged as waste water, since in water and at ambient temperature, almost 90% of cocaine is degraded in 24 h. (Gheorghe et al., 2008). At the same time, the untreated control group only received tap water and the vehicle control group only received ethanol at the same concentration (0.000333%) as the cocaine-exposed eels. The treatment lasted for fifty days. At the end of the exposure period, we tested whether the eels exposed to cocaine were able to recover from the changes induced by the drug. For this, two of the three cocaine-exposed groups were kept in cocaine-free tap water for three and ten days prior to sacrifice. At the end of the experimental period, the eels were anesthetised using MS-222 at a concentration of 100 mg L^{-1}, weighed and measured. Blood was taken from the posterior cardinal vein with a 5 mL syringe; once coagulation took place in Eppendorf tubes for 2–4 h, blood was centrifuged for 15 min at 2000 g, and serum was collected and stored at −22 °C until alanine aminotransferase (ALT) and C-reactive protein (CRP) analysis. Blood glucose was evaluated immediately on whole blood using the Glucometer Elite XL (Bayer, IN, USA), measuring concentrations of 1.1–33.3 mmol L^{-1} glucose. After blood collection, the animals were killed by decapitation. From each animal, samples of liver and posterior, mesonephric kidneys were taken and 1) processed for light microscopy, for the evaluation of general morphology; 2) weighed, frozen in liquid nitrogen and stored at −80 °C until the analysis of cytochrome oxidase and caspase-3 activities, and GRP-78 levels.

2.4. Histology

To evaluate the general organ morphology, samples of liver and kidney were fixed at room temperature in Bouin's fixative for 24 h, dehydrated in ascending alcohols, cleared in Histolemon, embedded in Paraplast and cut into 6 μm serial sections. The sections were subjected to routine histological analysis and stained with Mallory trichromic stain. Observations were performed by means of a Zeiss Axioskop microscope (Carl Zeiss MicroImaging s.p.a., Milan, Italy), connected to a camera attached to an IBM computer running the Kontron Elektronik KS 300 image analysis system (Carl Zeiss MicroImaging s.p.a., Milan, Italy) and Adobe Photoshop.

2.5. Cytochrome oxidase (COX) activity

Measurement of COX activity was performed polarographically at 25 °C with a Clark-type electrode in media containing 30 μM cytochrome c, 4 μM rotenone, 0.5 mM dinitrophenol, 10 mM Na-malonate, 75 mM HEPES, pH 7.4 [59]. COX activity was measured in the whole organ homogenate; 100 mg of liver or kidney were diluted in Chappel and Perry medium (1 mM ATP, 100 mM KCl, 5 mM MgCl2, 1 mM EDTA, 5 mM EGTA, 50 mM HEPES pH 7.4) containing Lubrol PX (225 μg/mg protein) and incubated for 30 min in ice to unmask the activity of the enzyme. After incubation, COX activity was measured as oxygen

consumed in the presence of 4 mM ascorbate + 0.3 mM tetramethyl-p-phenylenediammine (TMPD) (Lionetti et al., 2004).

2.6. Detection of apoptosis

The evaluation of caspase-3 in liver or kidney was used as a marker of apoptosis. Tissues were homogenized in cold lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM dithiothreitol, 1,5 μM pepstatin) using the T10 basic ULTRA-TURRAX S10N-5G. The tissue debris were removed by centrifuging the homogenates at 16,000 g for 30 min at 4 °C. Protein content was measured by the Harthree-Lowry Protein Assay (Hartree, 1972). The Colorimetric Caspase 3 Assay Kit (Sigma-Aldrich, Milan, Italy) was used to detect caspase-3 activity, according to the manufacturer's protocol. Proteins were incubated for 2 h at 37 °C in a reaction mixture containing the caspase-3 substrate acetyl-Asp-Glu-Val-Asp (200 μM) labelled with p-nitroanilide, pNA. From the hydrolysis of the labeled substrate, free p-NA was released, absorbing at a wavelength of 405 nm. The activity of caspase-3 was expressed as nmol of free pNA normalized for μgram of proteins and time (min).

2.7. GRP-78 levels

The expression of GRP-78 in liver tissues was evaluated by Western blotting. Homogenates were prepared in cold lysis buffer (50 M Tris-HCl, pH 6.8, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1 mM EDTA, 1% glycerol, 1% PMSF, and an inhibitor cocktail, by using the T10 basic ULTRA-TURRAX S10N-5G). Homogenates were centrifuged and the protein content was determined as described in previous section. Proteins (50 μg) from each sample were mixed with Laemmli loading buffer and loaded onto a 10% sodium dodecyl sulfate polyacrylamide gel. After the electrophoretic separation, proteins were transferred to a polyvinylidene fluoride membrane (Millipore Corp. Bedford, MA, USA). Then the membrane was treated for 1 h with a blocking solution consisting of 5% milk in Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl) and incubated overnight at 4 °C with an anti-GRP 78 antibody raised in rabbit (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:1000 in 0.1% milk in Tris-buffered saline plus 0.1% Tween-20. Finally the membrane was incubated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Milan, Italy). Immunocomplexes were revealed using a chemiluminescence detection kit (Millipore Corp.) according to the manufacturer's instructions. For normalization, a mouse anti-GAPDH antibody (Santa Cruz Biotechnology Inc.) was used.

2.8. Serum enzymes

Alanine aminotransferase (EC 2.6.1.2; L-Alanine: 2-Oxoglutarate Aminotransferase, ALT or A1aAT; Glutamate Pyruvate Transaminase, GPT) and C-reactive protein (CRP) were determined with the aid of commercial kits (GPT/ALT FL and CRP-FL; Chema-Diagnostica, Monsano, AN, Italy). The principle of the ALT assay is as follows: ALT catalyzes the transaminase reaction between L-Alanine and 2-Oxoglutarate. The pyruvate formed is reduced to lactate in the presence of lactate dehydrogenase (LDH). As the reactions proceed, NADH is oxidized to NAD^+. The disappearance of NADH per unit time is followed by measuring the decrease in absorbance at 340 nm. The principle of the CRP assay is as follows: CRP selectively reacts with an anti-CRP antibody and forms an immunocomplex. The turbidity produced is proportional to the concentration of CRP in the sample, and can be measured at the wavelength of 340 nm. The changes in absorbance at 340 nm (ΔA/min) were measured by using the Smart Spec Plus Spectrophotometer (Bio-Rad Laboratories). The ALT activity was expressed as UI L^{-1}, multiplying the ΔA/min by the factor indicated from the producer. The CRP activity was expressed in mg L^{-1}. The sensitivity/limit of detection were 0.169 L^{-1} and 1.0 mg L^{-1}, respectively.

2.9. Statistical analysis

The quantitative data were subjected to statistical analysis; the values were expressed as means ± standard deviation of mean (SD). All the data were first tested for normality and homogeneity of variance to meet statistical assumptions; the Bartlett test was used to verify the homogeneity of variance. The data were compared by one-way analysis of variance (ANOVA), followed by the Tukey-Kramer multiple comparison test. Commercial software (SigmaStat Version 4.0; SPSS) was used to perform all statistical analyses; differences were considered significant when $P < 0.05$.

3. Results

Ethanol exposure did non affect any of the parameters evaluated; indeed, none of the vehicle (ethanol)-exposed eels showed differences compared to untreated control (tap water-exposed) eels (Figs. 1 and 2).

3.1. General morphology

The liver of control animals (Fig. 1A–C) was composed of branching and anastomosing, two cell thick cords of polygonal hepatocytes, corresponding to the typical morphology of the fish liver (Mumford et al., 2007). Sinusoids were irregularly distributed between the hepatocytes, having a distinctive central nucleus with densely staining chromatin margins, and a cytoplasm containing large amounts of lipids, as expected in the silver stage. After the exposure to cocaine, liver tissue showed architectural and structural alterations, such as necrotic areas, loss of parenchymal cells, hepatocytes devoid of lipid content, karyolysis and pyknotic nuclei (Fig. 1D–F). Three days (Fig. 1G) and ten days (Fig. 1H) after the interruption of cocaine exposure, livers showed signs of returning to the normal situation, with a gradual increase of lipid content.

The eel kidney is a paired organ, with a pronephric portion remaining separate as a lymphoid, haematopoietic structure containing few nephrons and a posterior kidney, a mesonephros, performing excretory and endocrine functions (Mumford et al., 2007). Fish nephrons consist of several segments with a specific structure: a glomerulus within Bowman's capsule; proximal tubules, with cells provided with an apical brush-border; distal tubules, without a brush-border; collecting tubules, without a brush-border, characterized by a thin layer of smooth muscle and connective tissue. PAS staining allows to distinguish, in the proximal tubules, a first (P1) segment, having cells with large spherical, pale-staining nuclei and a second (P2) segment, with centrally located nuclei and intensely eosinophilic cytoplasm (Watanabe and Takei, 2011). Moreover, melanomacrophage centers, containing melanin, which acts as a scavenger for free radicals, are present (Mumford et al., 2007). In the renal tissue of the control eels, the typical kidney morphology was seen: many glomeruli and renal tubules surrounded by the hematopoietic tissue; some melanomacrophage centers (Fig. 2A–C). After the exposure to cocaine (Fig. 2D–G), numerous melanomacrophage centers (Fig. 2D), dilated glomeruli and reduced Bowman's space were observed. The renal tubules appeared dilated and their epithelial cells showed signs of degeneration, as pyknotic nuclei and karyolysis (Fig. 2E–G). Three days (Fig. 2H) and ten days (Fig. 2I) after the interruption of cocaine exposure, the kidney still showed the alterations observed in the cocaine-exposed group.

3.2. Cytochrome oxidase (COX) activity

After the exposure to cocaine, liver COX activity increased significantly ($P = 0.01$) compared to control eels (Fig. 3). Three days after the interruption of cocaine exposure, liver COX activity decreased, remaining higher ($P = 0.03$) than in controls however, and only ten days after the interruption of cocaine exposure, did COX activity return to values similar to those of non-exposed eels ($P = 0.06$). After the

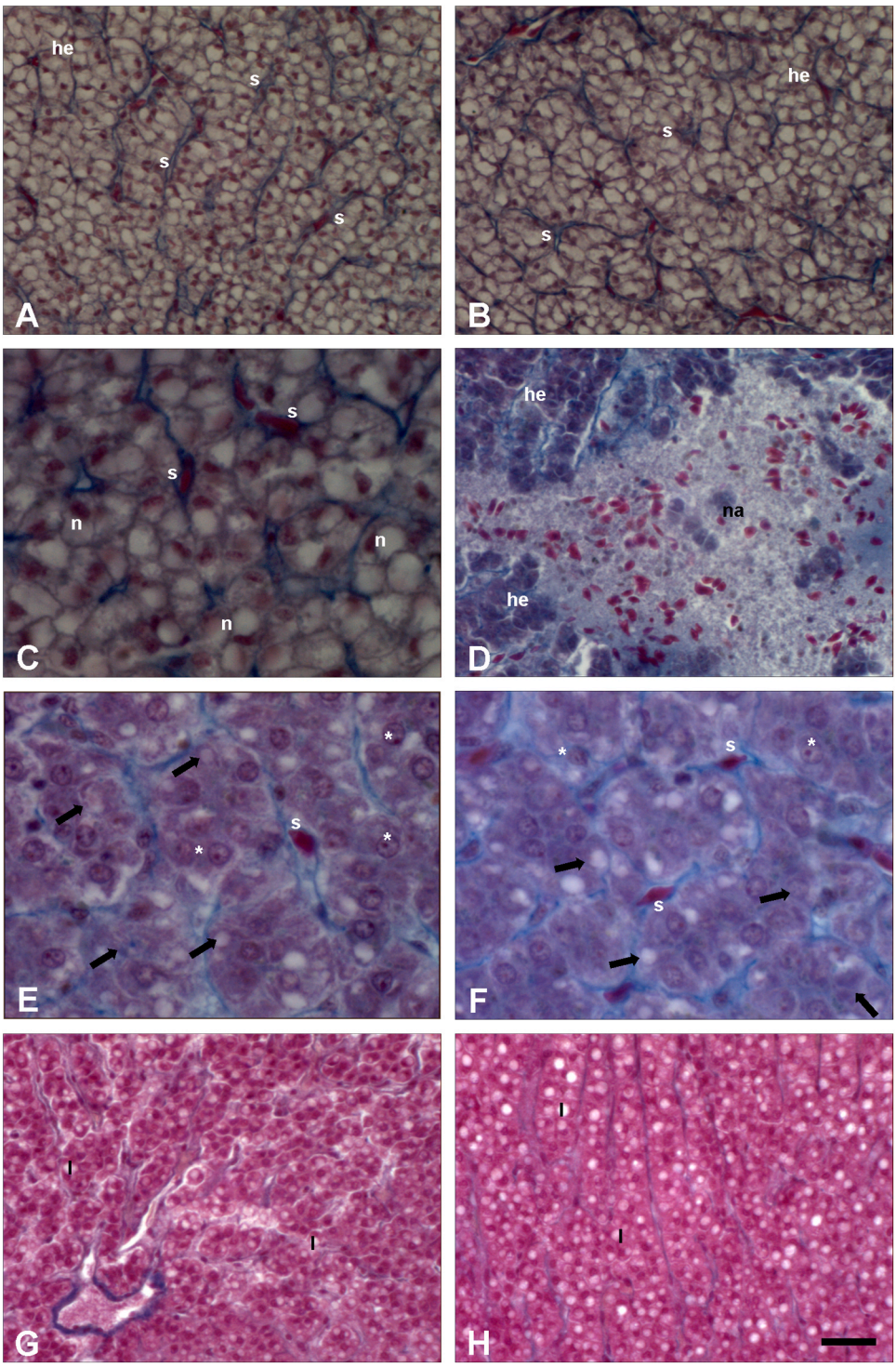

Fig. 1. Light micrographs of the liver of *Anguilla anguilla*. Mallory staining. A, C: control specimens (n = 30); B: vehicle control specimens (n = 30); D, E, F: exposed specimens (n = 30); G: post-exposure recovery three days (n = 30) and H: post-exposure recovery ten days (n = 30) specimens. (A, B, C) The hepatic parenchyma of the control eels showed polygonal hepatocytes (he) arranged in two cell thick cords, separated by sinusoids (s). The hepatocytes showed a nucleus (n) with densely staining chromatin margins; the cytoplasm contained large amounts of lipids (l). (D, E, F) After the exposure to cocaine, liver showed necrotic areas (na) and loss of parenchymal cells. The hepatocytes (he) showed karyolysis (arrow), pyknotic nuclei (asterisk) and loss of lipid (l) content. Three days (G) and ten days (H) after the interruption of cocaine exposure, the liver tissue showed signs of a return to a normal morphology, with a gradual increase of lipid (l) content. Scale bar: A , B, D, G, H: 20 μm; C, E, F: 8 μm.

exposure to cocaine, kidney COX activity increased significantly (P = 0.02) compared to control eels. Similar increases in COX activity were still observed three (P = 0.02) and ten (P = 0.029) days after the interruption of cocaine exposure (Fig. 3).

3.3. Caspase-3 activity

After the exposure to cocaine, a significant increase of both liver (P = 0.032) and kidney (P = 0.019) caspase-3 activity was found. Three days after the interruption of cocaine exposure, caspase-3 activity returned to a basal level (liver: P = 0.61; kidney: P = 0.69). Ten days after the interruption of cocaine exposure, caspase-3 activity appeared significantly (P = 0.012) lower than the control values in the liver, whereas it was similar to the control values in the kidney (P = 0.67) (Fig. 4).

3.4. GRP78 levels

The levels of GRP78 in the liver were investigated by Western blotting. Since no eel-specific GRP78 has been characterized, we used the protein sequence of zebrafish GRP78 to evaluate the similarity of the human and fish proteins. We found a sequence identity of 91.437%

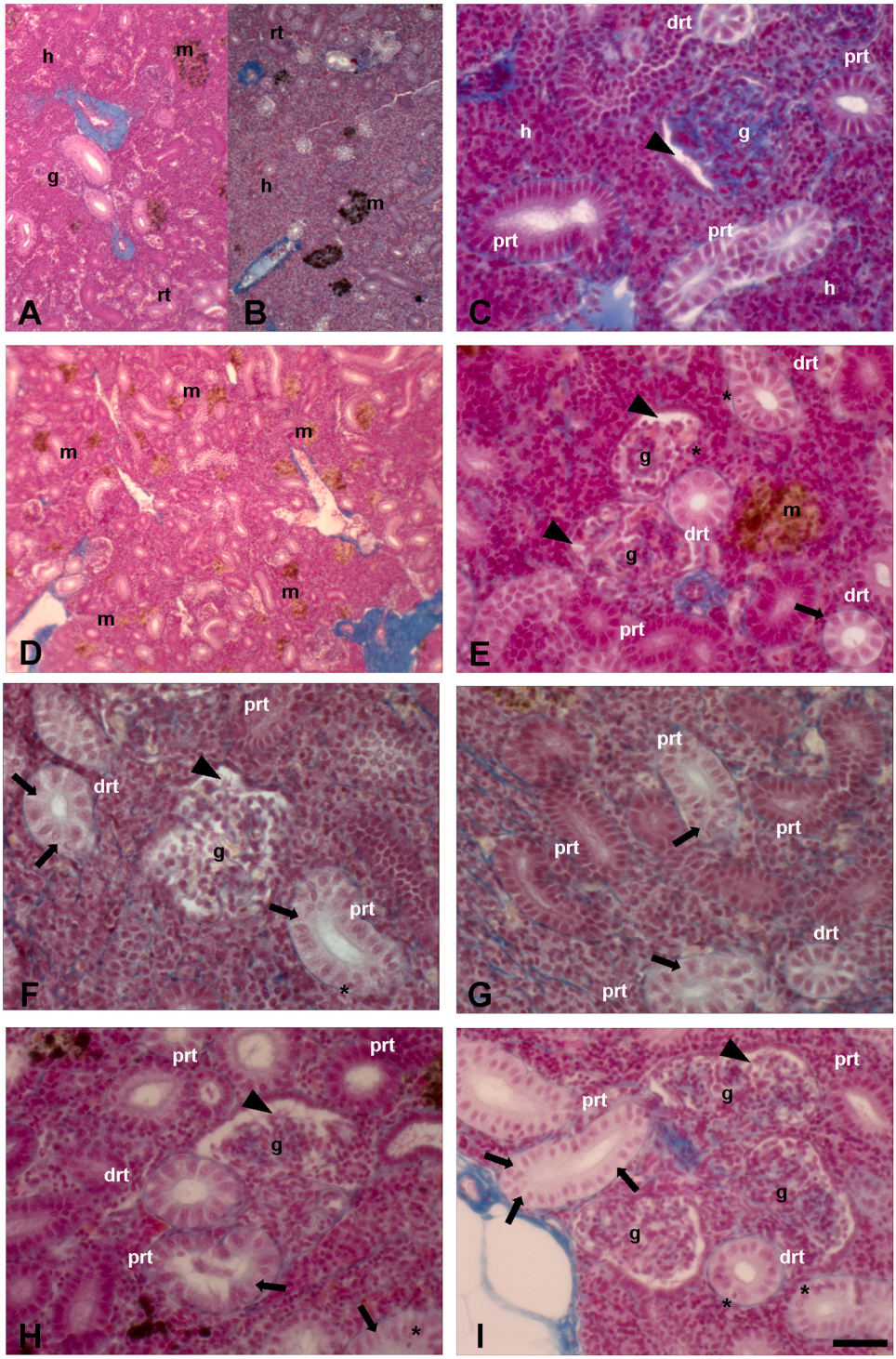

Fig. 2. Light micrographs of the kidney of *Anguilla anguilla*. Mallory staining. A, C: control specimens (n = 30); B: vehicle control specimens (n = 30); D, E, F, G: exposed specimens (n = 30); H: post-exposure recovery three days (n = 30) and I: post-exposure recovery ten days (n = 30) specimens. (A, B) The renal tissue of the control eels showed glomeruli (g), renal tubules (rt) ands melanomacrophage (m) centers surrounded by the hematopoietic (h) tissue. (C) At higher magnification, it was possible to distinguish the Bowman's space (arrowhead) of the glomerulus (g); the proximal (prt) and the distal (drt) tubules. (D, E, F, G) After the exposure to cocaine, in the renal tissue, many melanomacrophage (m) centers; dilated glomeruli (g) and reduced Bowman's space (arrowhead); pyknotic nuclei (asterisk) and karyolysis (arrow) were observed. (H, I) Three days (H) and ten days (I) after the interruption of cocaine exposure, the kidney still showed the alterations observed in the exposed group. Scale bar: A,B: 80 μm; C, D, E, F, G, H, I: 20 μm.

(Uniprot database), thus we were confident that an anti-GRP78 antibody raised against the human GRP78 would recognize the eel protein (Fig. 5). Indeed Western blot analyses revealed bands of the expected molecular weight (72 kDa). GRP78 expression increased slightly (P = 0.059) in cocaine-exposed liver samples and continued to increase three days after the interruption of cocaine exposure, when its levels became much higher than controls (P = 0.007) (Fig. 5). Ten days after the interruption of cocaine exposure, GRP78 expression decreased but its levels were still higher (P = 0.008) than control specimens. In conclusion, GRP78 was more expressed in post-exposure recovery than in cocaine-exposed specimens.

3.5. Blood glucose levels

After the exposure to cocaine, a significant (P = 0.01) increase in blood glucose levels was observed. Three (P = 0.015) and ten (P = 0.009) days after the interruption of cocaine exposure, the levels were still significantly higher than control ones (Fig. 6).

3.6. Serum enzymes

After the exposure to cocaine, large increases in the serum levels of ALT (Fig. 7A) and CRP (Fig. 7B) were observed. Three and ten days after

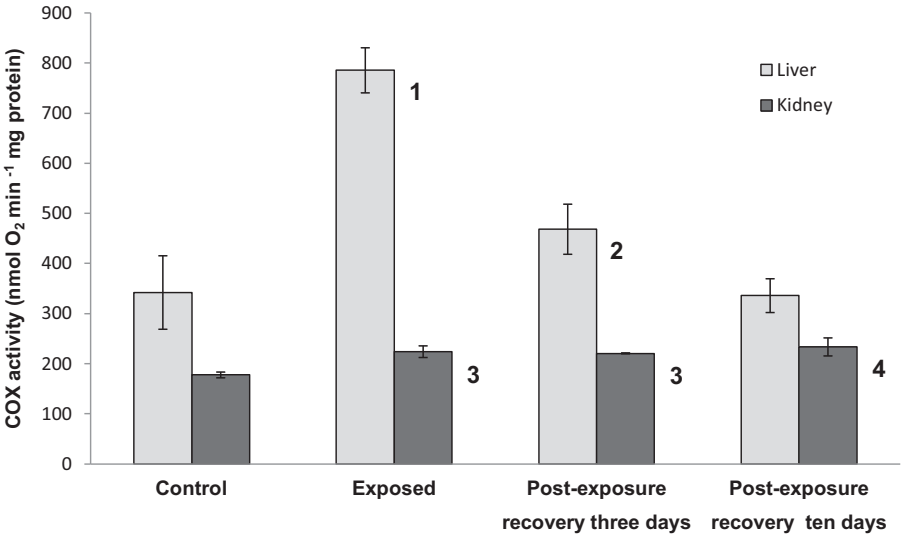

Fig. 3. Cytochrome oxydase (COX) activity in control (n = 30), exposed (n = 30), post-exposure recovery three (n = 30) and ten (n = 30) days specimens. Values are expressed as nmol O_2/min x mg protein. One-way ANOVA, followed by the Tukey-Kramer multiple comparison test. Values are mean ± SD of the mean. 1: significantly (P = 0.01) different from the control values. 2: significantly (P = 0.03) different from the control values. 3: significantly (P = 0.02) different from the control values. 4:significantly (P = 0.029) different from the control values.

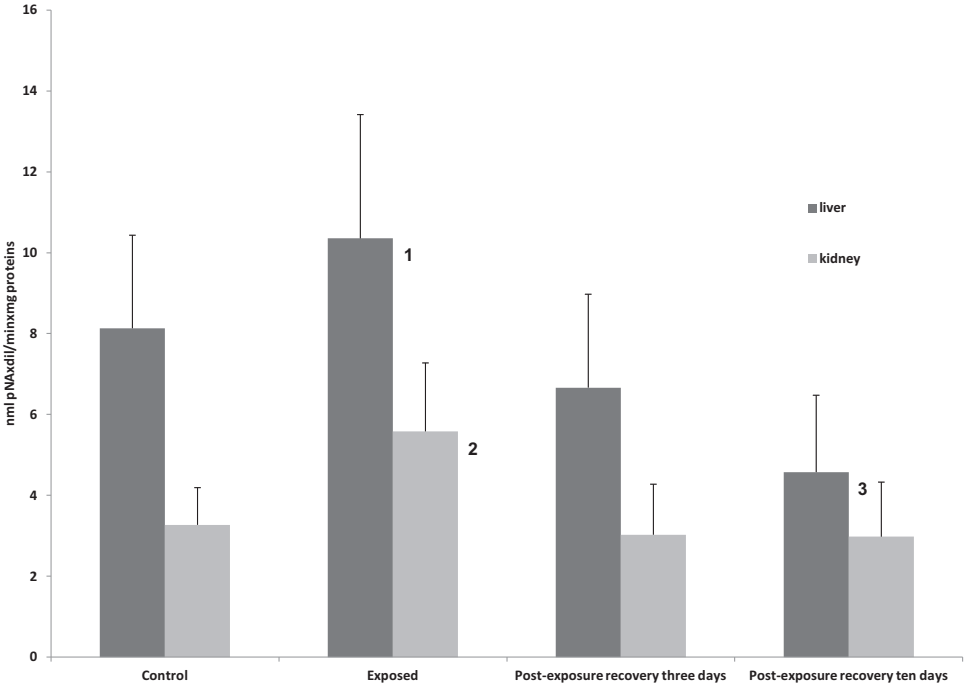

Fig. 4. Caspase-3 activity in liver and kidney of control (n = 30), exposed (n = 30), post-exposure recovery three (n = 30) and ten (n = 30) days specimens. Values are expressed as nmol of free pNA normalized for μg of proteins and time (min). One-way ANOVA, followed by the Tukey-Kramer multiple comparison test.Values are the mean ± SD of the mean. 1: Significantly (P = 0.032) different from the control values. 2: Significantly (P = 0.019) different from the control values. 3: Significantly (P = 0.012) different from the control values.

the interruption of cocaine exposure, the levels of both enzymes decreased, remaining however higher than in controls.

4. Discussion

Our present results show, for the first time, that environmental cocaine concentrations damage the liver and the kidney of the European eel, inducing alterations that often persist after the interruption of cocaine exposure.

As regards the general morphology, the routine histological analysis showed the presence of nuclear alterations, such as karyolysis and pyknotic nuclei in both the liver and the kidney. Moreover, cytoarchitectural and structural alterations were observed, such as necrotic areas, loss of parenchymal cells and hepatocytes devoid of lipid content in the liver; dilated glomeruli with reduced Bowman's space, dilated renal tubules and the appearance of numerous melanomacrophage centers in the kidney. This last observation, the increased presence of melanomacrophage centers in the kidney of cocaine-exposed eels, agrees with the increase in the number of these centers in cichlid fish exposed to the antidepressant, fluoxetine (Rey Vàzquez et al., 2020) and with the increase in size or frequency of these centres observed in fish in conditions of environmental stress, confirming the utility of MMCs as general indicators of exposure of fish to contaminated environments (Passantino et al., 2020). It should be noted that in this study the kidney appeared to be more sensitive to the effects of cocaine than the liver, since three and ten days after the interruption of cocaine exposure, the kidney still showed alterations whereas the liver showed signs of a gradual return to the normal situation.

The histopathological changes observed in cocaine-exposed eels may be classified as regressive changes, correlated to a functional reduction of an organ (Bernet et al., 1999) and agree with the hepatotoxicity and the nephrotoxicity induced by cocaine in humans, in mammals and in

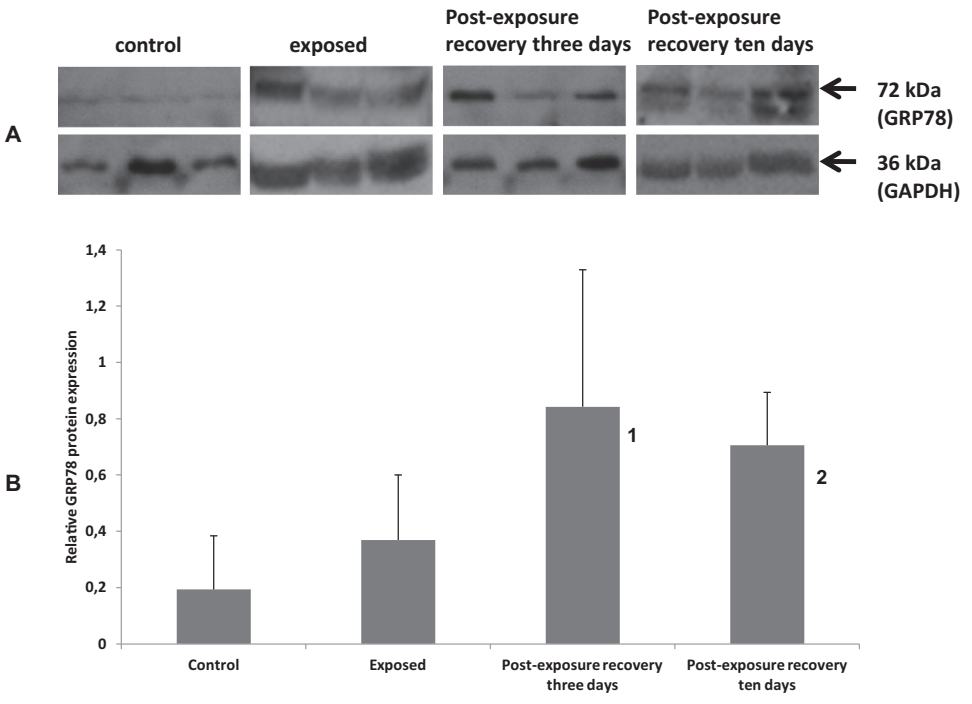

Fig. 5. GRP78 expression in control (n = 30), exposed (n = 30), post-exposure recovery three (n = 30) and ten (n = 30) days specimens. (A) Representative Western blots showing GRP78 expression in liver homogenates of three different specimens for each group. (B) Band intensities of all samples was normalized towards band intensities of the house-keeping protein GAPDH. One-way ANOVA, followed by the Tukey-Kramer multiple comparison test. Values are the mean ± SD of the mean. 1: Significantly (P = 0.007) different from the control values. 2: Significantly (P = 0.008) different from the control values.

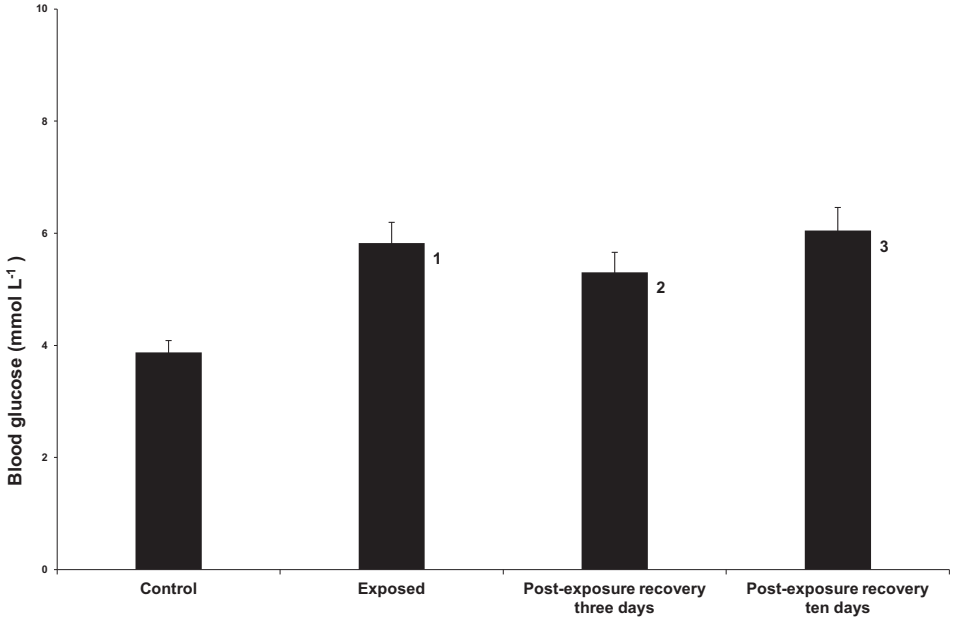

Fig. 6. Blood glucose levels in control (n = 30), exposed (n = 30), post-exposure recovery three (n = 30) and ten (n = 30) days specimens. Values are expressed as nmol L^{-1}. One-way ANOVA, followed by the Tukey−Kramer multiple comparison test. Values are the mean ± SD of the mean. 1: Significantly (P = 0.01) different from the control values. 2: Significantly (P = 0.015) different from the control values. 3: significantly (P = 0.009) different from the control values.

fish (Arinc and Bozcaarmutlu, 2003; Valente et al., 2012). Cocaine is metabolized mainly by plasma and liver esterases into benzoylecgonine (BE), ecgonine (E), and ecgonine methyl ester (EME); only a minor part of cocaine is N -demethylated to norcocaine (NCOC) by isozymes of the cytochrome P-450 (CYP) superfamily. NCOC can in turn be oxidized to N-hydroxynorcocaine and then to the free radical norcocaine nitroxide. As this compound can be easily backreduced in cells, it has been proposed that this redox cycle may result in the sustained production of reactive oxygen species (ROS), with subsequent oxidative stress (Valente et al., 2012). Cocaine-induced hepatotoxicity has been associated with NCOC and N-hydroxynorcocaine production, and the direct oxidative damage by ROS generated by this redox cycling during the metabolic cascade (Valente et al., 2012). This mechanism has been shown also in the mullet (*Liza saliens*), where P4503A and P4502B isozymes were found to be responsible for cocaine-induced hepatotoxicity (Arinc and Bozcaarmutlu, 2003). Thus, it is possible that a similar mechanism effect occurs in the liver of cocaine-exposed eels. As regards cocaine-induced nephrotoxicity, in most reported cases, acute renal failure was associated with rhabdomyolysis, hyperthermia or ischemia due to vasoconstriction (Valente et al., 2012). An effect of rhabdomyolysis is the

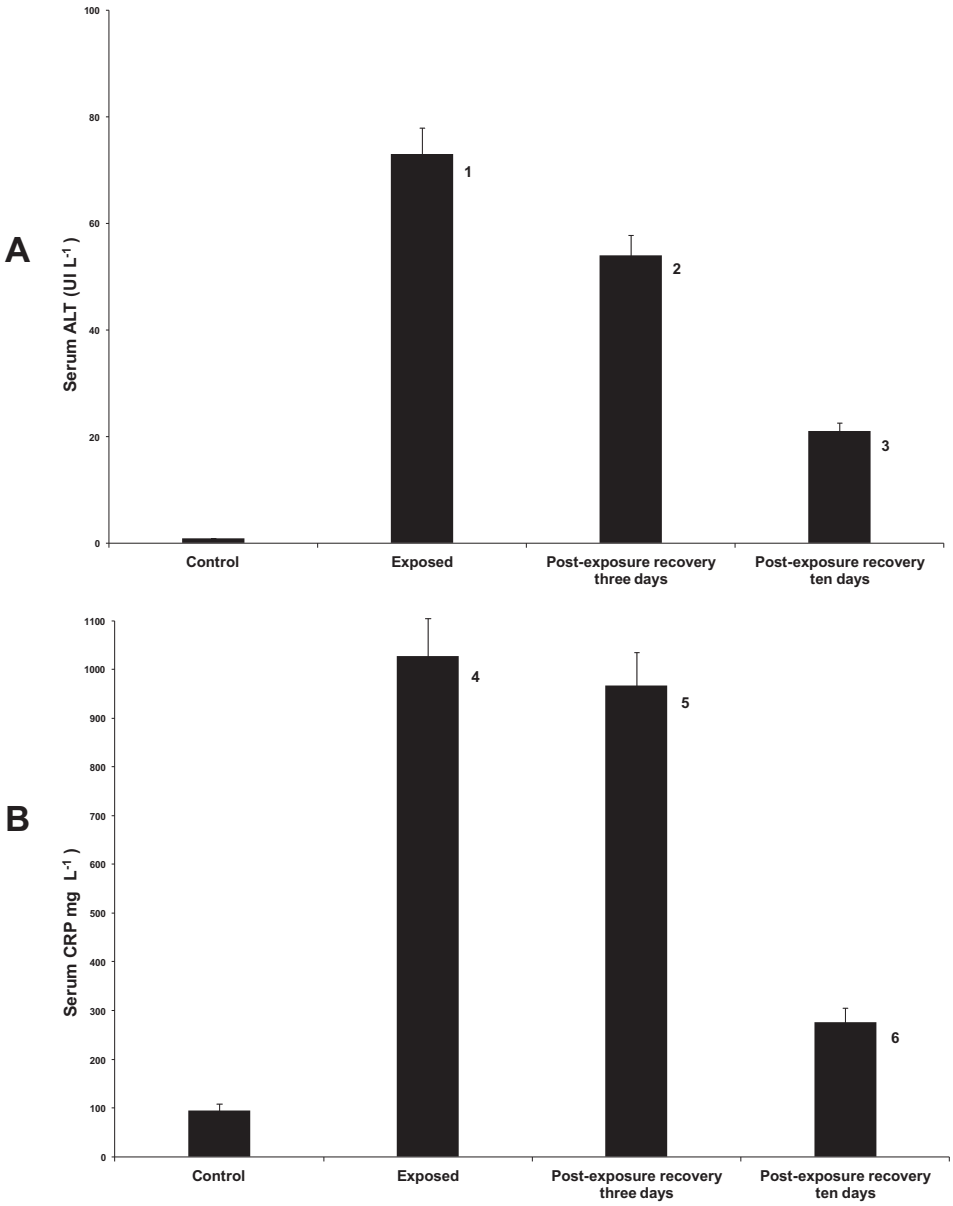

Fig. 7. (A) Serum alanine aminotransferase (ALT) and (B) serum C-reactive protein (CRP) levels in control (n = 30), exposed (n = 30), post-exposure recovery three (n = 30) and ten (n = 30) days specimens. Values are expressed as UI L^{-1} and mg L^{-1} , respectively. One-way ANOVA, followed by the Tukey-Kramer multiple comparison test. Values are the mean ± SD of the mean. 1: Significantly (P = 0.001) different from the control values. 2: Significantly (P = 0.039) different from the control values. 3: Significantly (P = 0.0031) different from the control values. 4: Significantly (P = 0.015) different from the control values. 5: Significantly (P = 0.021) different from the control values. 6: Significantly (P = 0.036) different from the control values.

leakage of large quantities of myoglobin into the circulation; when myoglobin levels exceed the protein-binding capacity of the plasma, it precipitates in the glomerular filtrate, causing cast formation and the accumulation of iron in the proximal renal tubules, with intratubular obstruction and proximal tubular cell injury (Khan, 2009). In our previous study of the effects of cocaine on the eel skeletal muscle (Capaldo et al., 2018), we found changes in this tissue that were reminiscent of rhabdomyolysis. Therefore, it is possible that the changes observed in the kidney of cocaine-exposed eels may be, in part, correlated to muscle injury; however, more in-depth studies are necessary to evaluate this hypothesis. Alternatively, cocaine may exert direct cytotoxic effects on the kidney, where cocaine is metabolized to form EME and to a minor extent, NCOC (Valente et al., 2012). It was suggested that NCOC underwent a subsequent oxidation to N-hydroxynorcocaine through CYP3A4, as described in the liver. NCOC was found to be a more potent nephrotoxic compound than cocaine itself, inducing in primary cultured human proximal tubular epithelial cells of the kidney death according an apoptotic pattern (Valente et al., 2012). It is possible that the same processes are responsible for the changes observed in the kidney of the eels exposed to cocaine.

As regards the COX and caspase activities, and GRP78 expression, a significant increase in COX activity was observed after cocaine exposure in both the liver and kidney. During the recovery period, in the liver, COX activity tended to decrease and it returned to control values ten days after the interruption of cocaine exposure, whereas in the kidney, COX activity was still elevated ten days after the interruption of cocaine exposure. The increase in COX activity, observed in the eels after cocaine exposure, is in contrast to the finding of decreased COX activity induced by cocaine in the rat prefrontal cortex (Vélez-Hernández et al., 2014). Several reasons may explain this difference: different animal/cellular models; different doses and times of exposure to cocaine and probably the different role played by the liver and kidney (both involved in detoxification processes) compared to the prefrontal cortex. COX enzyme is a well-known biomarker of oxidative metabolism (Lee and Hüttemann, 2014), since it catalyzes the final step in the mitochondrial electron transfer chain (ETC) and the reduction of oxygen to water, producing most of the cellular energy. The observed increase in COX activity suggests an increase in specific mitochondrial enzyme activity and/or in mitochondrial protein mass. Since the liver and the kidney are involved in metabolism and the subsequent excretion of xenobiotics

(Bernet et al., 1999), the increase in COX activity observed after cocaine exposure may reflect the increased energy requirements for the detoxification process. COX hyperactivity could lead in turn to mitochondrial ROS generation and activate the intrinsic apoptotic pathway, committing the cell to programmed death (Hüttemann et al., 2012). This hypothesis is in accordance with the increase in the activity of caspase-3, the major executioner caspase in the apoptotic pathway (Brentnall et al., 2013), observed in both the liver and the kidney of the eels after cocaine exposure. Cocaine is known to induce caspase activation and apoptosis through a mechanism involving the release of cytochrome c from the mitochondria into the cytosol, and the subsequent activation of caspase-9 and caspase-3 (Dey et al., 2007). Our results, showing an increase in caspase-3 activity in the eels exposed to cocaine, agree with these data. Three days after the interruption of cocaine exposure, the caspase-3 activity returned to basal levels in both the liver and kidney. Subsequently, ten days after the interruption of cocaine exposure, in the liver, caspase-3 activity decreased to lower levels than controls, whereas it remained unchanged in the kidney. To understand better the trend of the liver caspase-3 activity, we evaluated the expression of GRP78 in the liver. Indeed, GRP78, in addition to being the main marker of endoplasmic reticulum (ER)-stress (Hotamisligil, 2010), acts as an apoptotic regulator by protecting the host cell against ER stress-induced cell death. GRP78 inhibits cytochrome c-mediated caspase activation, and expression of GRP78 blocks both caspase activation and caspase-mediated cell death. Our results showed a slight increase in GRP78 expression after cocaine exposure, and a more marked increase in post-exposure recovery specimens. Comparing the trend of GRP78 expression to that of caspase-3 activity, we found that the highest levels of liver GRP78 expression, observed in the post-exposure recovery eels, corresponded to the lowest levels of liver caspase-3 activity, observed in the same specimens. Although other processes cannot be excluded, this suggests that the level of caspase-3 activity reflects an inhibition of caspase-3 by GRP78. The increase in GRP78 expression in the liver suggests the presence of ER stress concomitant to tissue damage. The highest levels of GRP78 expression, observed after the interruption of cocaine exposure, could be explained by the role of GRP78 as key regulator of the pro-survival arm of the unfolded protein response (Pfaffenbach and Lee, 2011). Thus, when cocaine was removed, liver cells promoted GRP78 expression in order to restore homeostasis and to counteract apoptosis and consequent tissue damage.

As regards the blood glucose levels, a consistent increase in blood glucose levels was observed in both cocaine-exposed and post-exposure recovery specimens. Blood glucose is considered to be a classical marker of a stress response, since glucose provides energy substrates to vital organs and thereby to cope with the increased, stress-related, energy demand. Blood glucose increase is considered to be a general response of fish to acute pollutant effects (Banaee, 2013); a significant glucose increase was reported in *A. anguilla* exposed to sub-lethal concentrations of diazinon (Ceron et al., 1997) and in the catfish *Pangasianodon hypophthalmus* exposed to chromium (Majharul Islam et al., 2020). Our results, showing an increase in blood glucose levels in both cocaine-exposed and post-exposure recovery specimens, agree with these data, and confirm that cocaine behaves like a real pollutant, inducing stress in the eels. Cocaine is known to increase plasma glucose levels through catecholamine release (Argente Villaplana et al., 2008); it is possible that such a mechanism took place also in the eels, where a consistent increase in plasma catecholamines was observed after cocaine exposure (Gay et al., 2013).

As regards the serum enzymes, the serum levels of ALT and CRP strongly increased after cocaine exposure. Although a decrease was observed after the interruption of cocaine exposure, the ALT and CRP serum levels observed remained higher than the control levels. ALT enzyme is a well-established serum marker of liver function, and its increase may be an index of liver injury. ALT catalyzes the transaminase reaction between L-alanine and 2-oxoglutarate, forming pyruvate, which is an important contributor to the citric acid cycle, providing energy to the cells. Together with aspartate aminotransferase (AST), ALT is highly concentrated in the liver; since, however, AST is also diffusely represented in the heart, skeletal muscle, kidney, brain, and ALT has low concentrations in skeletal muscle and kidney, an increase in ALT serum levels is more specific for liver damage (Giannini et al., 2005). The increase of ALT serum levels observed in cocaine-exposed and post-exposure recovery eels confirms the morphological observations showing alterations of the liver structure, and agrees with ALT increases observed in mice after exposure to cocaine (Mai et al., 2019); in *A. anguilla* after exposure to propanil (Sancho et al., 2009) and in humans after exposure to respirable suspended particulate matter and nitrogen dioxide (Dey et al., 2015). CRP is an evolutionary conserved protein, primarily synthesized by liver hepatocytes and secreted in the plasma, and it is an important constituent of innate immunity of the host (Ansar and Ghosh, 2013). CRP has been found in different species from arthropods to humans, and in 17 different species of fish, including *A. anguilla* (Pathak and Agrawal, 2019). CRP is considered to be a bioindicator of the health of fish; its levels increase in response to bacterial pathogens, xenobiotics and anti-ectoparasitic chemicals (Kodama et al., 2004; Liu et al., 2004). The increase of CRP serum levels observed in cocaine-exposed and post-exposure recovery eels confirms that cocaine induced a condition of disease in the eels, and agrees with the increase of CRP levels observed after cocaine usage (Siegel et al., 2002).

5. Conclusion

The morphological and biochemical analyses showed that cocaine, even at low environmental concentrations, induced metabolic and morphological changes in the liver and the kidney of the eel. The European eel is under threat and in serious decline, due to many causes, including overfishing, habitat loss, presence of parasites, climate change and water pollution. Given the role of the liver and the kidney in the physiology of the organism, one can assume that environmental cocaine could worsen the serious state of decline of this species. However, our results also show improvements of many physiological and morphological parameters, after the interruption of cocaine exposure, suggesting that a proper environmental clean-up policy would safeguard this species. Our present study shows, for the first time, that environmental cocaine concentrations damage the liver and the kidney of the European eel, inducing alterations that often persist after the interruption of cocaine exposure.

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CRediT authorship contribution statement

Anna Capaldo: Conceptualization, Writing - review & editing, Methodology, Visualization. **Flaminia Gay:** Resources. **Ivana Caputo:** Conceptualization, Writing - original draft preparation, Visualization. **Lillà Lionetti:** Conceptualization, Writing - original draft preparation, Visualization. **Gaetana Paolella:** Investigation, Resources. **Ilaria Di Gregorio:** Investigation, Resources. **Stefania Martucciello:** Investigation, Resources. **Mariana Di Lorenzo:** Investigation, Data curation. **Luigi Rosati:** Formal analysis, Validation. **Vincenza Laforgia:** Conceptualization , Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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