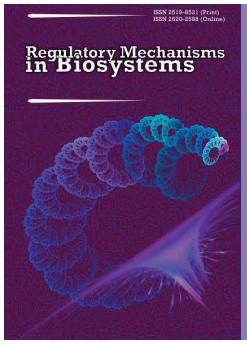

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Sublethal doses of copper sulphate initiate deregulation of glial cytoskeleton, NF-kB and PARP expression in *Capoeta umbla* brain tissue

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Copper sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$) is widely used as a pesticide not only in agricultural but in aquaculture farming as well. Copper sulphate is a cheap chemical and able to contaminate the environment, especially water sources, which is crucial for fish harvesting and farming. The copper contamination in some areas is caused over decades because this pesticide has long been used everywhere. Copper ions inhibit invasive aquatic plants and many microorganisms but contaminate soil and natural water resources. The family of copper-containing chemicals is frequently used as algaecides in swimming pools. Despite the high toxicity of copper ions for fish in freshwater ponds, copper sulphate remains one of the prevalent pesticides in fish farming everywhere. High cytotoxicity and accumulation of the copper ions in sediments require study and calculation of the optimal dosage for its use as an antiseptic agent which will not have a detrimental effect on various tissue types of aquatic organisms. The main recognized mechanism which accompanies the toxic effect of copper ions is the generation of oxidative stress. Neural tissue cells are extremely susceptible to oxidative damage and the functions of the CNS are critical to the vitality of organisms. Glial cells maintain the structure and many vital functions of neurons. The cytoskeleton glial fibrillary acidic protein (GFAP), transcriptional nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and Poly(ADP-ribose) polymerase (PARP) are critical participants in a cellular response to a toxic agent impact. As this takes place, it could be applied in biomarking of heavy metal toxicity. In the presented study, we investigated the effects of copper ions on PARP, NF-kB, and GFAP expression in the Tigris scraper *Capoeta umbla* brain tissue. For 96 hours the fish were exposed to copper sulphate at sublethal concentrations, namely 1/2, 1/4 and 1/8 of the LD_{50} value. Western blot analysis of GFAP and PARP was used to assess further effects in the brain tissue. Every studied dose of copper significantly downregulated the expression of GFAP after 72 hours of treatment. In spite of the common increment in the GFAP content, 48 hours exposure to copper initiated the upregulation of that cytoskeleton marker. Moreover, treatment with copper sulphate induced several changes in the β-actin level, especially in the fish group treated for 72 hours. The observed effect of copper in the fish brain evidences the unspecific toxic effect of the copper ions in the brain tissue cells. The obtained results demonstrated meaningful disturbance in the expression of transcriptional factor NF-kB in the brain of the fish group exposed to copper. The changes found in the fish brain indicate the dose-dependent effect in a concentration range 185–740 µg/L of copper sulphate during 72 hours. However, the exposure to low dose of copper ions showed no effect in the fish group treated for 24 hours. Comparative analyses of the PARP content in the brain of fish exposed to copper for 72 hours was significantly less than in the groups treated with copper for both 24 and 48 hours. Thus, the copper ions in the dose range 185–740 µg/L can suppress PARP expression in a time-dependent manner. The results showed that copper ions could induce astroglial response accompanied by modulations of NF-kB and PARP-1 expression. The data obtained in this study suggest that copper sulphate has a significant effect on astrogliosis and DNA damage in the fish brain.

Keywords: pesticide; fish brain; astrocytes; transcriptional regulation; GFAP; DNA damage.

Introduction

Copper is considered to be a toxic pollutant for fish as well as one of the most widespread heavy metals of anthropogenic origin in freshwater ecosystems throughout the world (Frías-Espericueta et al., 2011). Agriculture and industrial enterprises are the main sources of environmental contamination by different copper compounds (Calomeni et al., 2018). Continuous advance in agricultural production has caused the growth of the number of freshwater systems that are exposed to various toxic contaminants including copper-bearing pesticides. Toxic pollutants can easily enter into rivers and lakes through minor wastewater flows. Copper sulphate ($CuSO_4$) is applied everywhere as a pesticide against both fungal and protozoal infection in agriculture. Copper ions were detected in several aquatic freshwater ecosystems and ground waters in concentrations within the limits of allowable level (Atabati et al., 2015). Over the last decade, aquaculture has been intensifying the output of products worldwide while there has been increased application of pesticides in agricultural fields (Kiaune & Singhasemanon, 2011). Food safety and environmental sustainability in industrialized advanced countries is one of the major problems in the world today. Fish production systems have been transformed from small ponds to intensive reservoirs of high stocking density and required use of chemicals to control fish diseases.

The widespread antiseptic effect of copper compounds allows it to be used as a common unspecific fungicide in various aquaculture and agricultural enterprises (Lasiene et al., 2016). Cu^{2+} containing pesticides are applied to purify water from algae, especially in shrimp ponds to remove filamentous algae (Chen & Lin, 2001). Moreover, $CuSO_4$ can

inactivate several toxic metabolites of cyanobacteria including geosmin and 2-methylisoborneol (Tucker & Hargreaves, 2003). The treatment of some widespread freshwater parasites, such as *Ichtyophthirius multifiliis*, with $CuSO_4$, requires high concentrations of this chemical, which are recomended at 50 μg/L minimal dose (Picon-Camacho et al., 2012).

Copper sulphate is one of the cheapest pesticides everywhere and this accounts for its leading position as a treatment for the defence of fish against microbial and external protozoan parasites in intensive fish farming (Han et al., 2001). In addition, copper sulphate is widely used in the aquaculture industry as an effective remedy of the prophylaxis and cure of various ectoparasitic and bacterial infections (Parks et al., 2018). Copper sulphate exposure on its own is an effective way of reducing the incidence of various fish parasite invasions including fungi, protozoa and trematodes (Reddy et al., 2004; Mitchell et al., 2008). Copper ions effectively inhibit the growth of several types of bacteria, including *Salmonella* spp., *Pasteurella* spp., *Vibrio* spp., etc. (Nouh & Selim, 2013; Lasiene et al., 2016). Long-term treatment with copper as well as a repetitive cycle of exposure can promote significant accumulation of this toxic element in aquaculture ponds. The concentration of copper in pond water remains at a toxic level at least 50 hours after single treatment and can also inhibit the fish growth (McNevin & Boyd, 2004). Furthermore, the fish can accumulate copper as was demonstrated in a catfish farm in Vietnam (Marcussen et al., 2014).

Moreover, some results have demonstrated the detrimental toxic effects of the copper sulphate exposure, which accompanied the antiseptic inhibition of infections in various ornamental fish including fish species of high commercial cost (Trivedi et al., 2012). Recently Wang et al. (2015) and colleagues showed that elevated levels of copper could initiate various cellular disturbances, especially intercellular contact damages, dysregulation of signalling pathways and cell viability in the fish gills.

The scrapers of the genus *Capoeta* Valenciennes, 1842 (Cyprinidae, Cypriniformes) is generally undemanding in terms of water conditions. *Capoeta* has a wide geographical range of distribution in Asia, including South China, North India, Afghanistan, Turkestan, the Aral Sea, the Middle East and Anatolia (Türkmen et al., 2002). Monitoring studies detected 18 *Capoeta* species in the inland waters of the Middle East (Coban et al., 2013). Among them, the Tigris scraper *Capoeta umbla* (Heckel, 1843) is one of the species that is overconsumed by people and has some economic value. *C. umbla* inhabits the Euphrates – Tigris river systems. It is also locally known as a "lake fish" or "stream fish" and it is the most commercially valued fish for the local people (Canpolat & Calta, 2001; Türkmen et al., 2002; Coban et al., 2013).

Recent data demonstrated that exposure to copper can induce oxidative stress and apoptosis in fish gills (Wang et al., 2015). In spite of the well studied toxicity of copper ions, the cytotoxic effects of different intake doses of copper in the fish brain remain unknown as well as the safe dosage of this chemical for fish farming. *C. umbla* is tolerant and adapted to varying environmental conditions (Coban et al., 2013).

Glial fibrillary acidic protein (GFAP) was first isolated as a senile plaques protein and first characterized in 1971 (Eng et al., 1971). Several GFAP isoforms are expressed in a vertebrate's brain, but GFAP-α isoform is dominant in the astroglial cells of the central nervous system (CNS) as a rule (Thomsen et al., 2013). GFAP is an intermediate filament protein that is expressed by only several glial cell types in the CNS. The expression of GFAP is limited with two general glial cell types, especially astrocytes and ependymal cells (Eng et al., 2000). GFAP is the most applicable cytoskeleton marker of astrocyte cells and its reactivation induced by different kind of injury. Astrocytes' intermediate filaments are involved in a wide range of crucial processes in the central nervous system, including cell response to toxic agents and the functioning of the blood-brain barrier (Nedzvetsky et al., 2006; Gliem et al., 2015).

GFAP has been shown as an important participant of the cell proliferation through the assembly-disassembly dynamic process of the filament network presented in the cells during mitosis. GFAP rearrangement can participate in astrocyte-neuron interactions as well as intercellular communication (Heimfarth et al., 2017). Moreover, GFAP is considered to be an important cytoskeleton component for repair of various central nervous system injuries (Middeldorp & Hol, 2011). One of the main roles of GFAP is to provide dynamic cytoskeleton rearrangement during astrocyte reactive response and formation of glial scars in injured areas of neural tissue in the main in both brain and retina (Yang & Wang, 2015; Tykhomyrov et al., 2016).

The key player in the cellular response is a transcriptional factor NF-kB. NF-kB mediated signalling is one of universal mechanisms of cell response regulation in various tissue types. The effect of copper on vital signalling pathways could switch the fate of cells to both survival of the cells and elimination of lethally damaged cells. McElwee et al. (2009) demonstrated in vitro that copper induces in liver cell culture a cellular response which is accompanied by NF-kB activation.

Every cell type requires mechanisms of repair of DNA damaged by genotoxic effects caused by environmental pollutants. Poly[ADP-ribose] polymerase (PARP) plays an important role in the repair of damaged DNA and participates in the regulation of cellular response to different toxic factors. In addition, PARP can regulate several critical transcriptiontion factors, including NF-kB, p53 and AP-1, which are involved in cellular response and cell reactivity when cells are exposed to external toxic factors (Martin et al., 2015; Zhao et al., 2016).

Taken together, the literature data on cytoskeleton rearrangement and modulation of transcriptional factors' activity, which provide the cells' viability, demonstrate the ultimate importance of the regulation of multifaceted cellular response for the maintenance of functions of brain cells exposed to copper ions. The mechanism of Cu ions' neurotoxicity remains insufficiently clear. Taking into account that many fish farms continue intensive use of copper sulphate as a pesticide, the study of molecular mechanisms of copper neurotoxicity is required to clarify its potential to modulate cellular reactivity in the brain of freshwater fish.

For this reason, the purpose of our study was to find out the role of glial cytoskeleton and transcriptional regulation in *C. umbla* neural tissue in course of cellular response to copper cytotoxicity.

Materials and methods

The performance of the experiment and exposure procedures were conducted in accordance with the Experimental Protocol approved by the Animal Experimentation Ethics Committee of Bingöl University.

Experimental animals and design. The *Capoeta umbla* (112 ± 11 g) individuals were selected from the Murat River, Bingöl, Turkey. The Murat River is a typical local freshwater system, 722 km long. It is a tributary of the Euphrates River in the South East Anatolia, Turkey. The Murat River occupies an area of the upper basin of the Euphrates and Tigris Rivers (Kirici et al., 2016). Selected fish were accommodated for adaptation into the aquaria at the Department of Fishery Science, Agricultural Faculty of Bingöl University. Each aquarium was a fibreglass tank of 600 L volume. The fish were lodged in two aquaria for three weeks in order to adapt before the start of the experiment. During the acclimation, the fishes were fed thrice daily with commercial fodder in a minimal amount of 2% of the body weight. The fish were kept without feeding for 24 h before starting the exposure to the copper sulphate and any kind of treatment. The following parameters of the water quality were fixed: alkalinity 42 ± 12 mg/L, total water hardness 175 ± 21 mg/L as $CaCO_3$, level of dissolved oxygen 8.42 ± 0.70 mg/L, pH 7.8 ± 0.6, and temperature 17 ± 4 °C.

To determine the dose-dependent toxic effect of copper ions, the fishes were divided into five groups and then treated with $CuSO_4 \cdot 5H_2O$ in a range of concentrations: 500, 1000, 1500, 2000 and 3000 μg/L. Each fish group consisting of randomly selected seven individuals was placed into an aerated 150 L tank and exposed to the specified concentration of copper sulphate for 96 h. The control of fish mortality was performed permanently and the dead fish were removed from the aquarium immediately.

The value LC_{50} was calculated with SPSS IBM computer soft as 1480 μg/L. On the basis of the determined LC_{50} value, three doses were chosen that accord to 12.5%, 25% and 50% of 96 h LC_{50} (185, 370, and 740 μg/L). The exposure to the above- mentioned doses was performed with various durations. Every 185, 370, and 740 μg/L group was separated into three subgroups containing seven fish (n = 7) which was treated with different time exposure to 24, 48, and 72 hours. The control group was formed of five individuals.

Protein samples' preparations. Freshly isolated brain tissue from the separate fish individuals was homogenized in a buffer at a ratio of tissue : buffer = 1 : 10 (weight : volume) in 25 mM tris-HCl (pH 7.4) containing 0.15 M NaCl, 2.0 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycoltetraacetic acid, 23 μM leupeptin, 6.5 μM aprotinin, 1.5 μM pepstatin A, 5 μg/ml soybean trypsin inhibitor, 1mM phenylmethylsulfonyl fluoride and 1 μM sodium orthovanadate. The homogenates were centrifuged at 20.000 g for 30 min at 4 °C. The supernatants were collected as cytosol protein fractions. The pellets were resuspended in the same buffer additionally containing 0.5% sodium dodecylsulphate. The samples were incubated for lysis of membrane and subcellular structures for 45 min at 4 °C. After the incubation, the samples were centrifuged at 20.000 g for 30 min at 4 °C. The collected supernatants contained insoluble protein fractions.

The protein concentration in each sample of protein extracts was determined by Bradford's method (Bradford, 1976). The samples were mixed in a ratio of 1 : 1 with Laemmli Sample Buffer containing 0.1 M dithiothreitol, and boiled for 5 min. Protein samples were frozen at –20 °C and stored before analysis for no more than two weeks.

Western blot. The protein samples (40 μg/track) were run in 10% denaturing gels and transferred onto PVDF membrane (INTRON Biotech., WEST-lott PVDF, cat. No. ITM-P3032) with 0.45 μm pore diameter. After transferring, the membranes were blocked in 5% w/v non-fat dried milk (Sigma-Aldrich, USA, cat. No. M7409) for 90 min at 30 °C and probed with anti-PAPR-1 (Abcam, cat. No. ab-194586), anti-NF-kB p65 (Abcam, cat. No. ab-16502), anti-GFAP (Santa Cruz, USA, cat. No. sc-9065), or anti-β-actin (Santa Cruz, USA, cat. No. sc-69879) antibodies diluted 1/2000 in Tris buffer saline containing 0.05% Tween-20 (v/v) (TBST) according to manufacturer's recommendations. After 12 hours incubation at 4 °C, the membranes were washed five times with TBST and then incubated with the corresponding anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma-Aldrich, USA, cat. No. A-0545) diluted 1/10000 in TBST for 60 min. Then, unbound antibodies were removed by 5-times washing in TBST. Immunoreactive bands were visualized using a high-performance chemiluminescence machine (G: Box Chemi-XX8 from Syngene, Synoptic Ltd. Cambridge, UK) with enhanced chemiluminescence plus Luminol (SC-2048, Santa Cruz Biotechnology (manufacturer). Densitometric analysis of the obtained results was performed using densitometry software Total Lab TL120 (USA) and normalized to the intensity of the respective β-actin bands. Molecular weights of stained polypeptide zones were identified by extrapolation of relative motilities of each polypeptide on a plot of prestained proteins with known molecular weight (PageRuler Prestained Protein Ladder, Fermentas, Germany, cat. No. 26616).

Immunohistochemistry. For immunohistochemical (IHC) detection of GFAP, the samples of fish brain were fixed in 4% para-formaldehyde for 24 h and washed in PBS for 1 h. The paraffin blocks of tissue samples were prepared after dehydration of fish brain in ethanol and xylene. Fixed slices (5 μm) were cut and dried at room temperature. After deparaffinization with xilol-ethanol, the slices were dried and rehydrated in PBS. Endogenous peroxidase activity was inactivated by incubation with solution of 10% methanol and 0.5% peroxide hydrogen in PBS for 15 min at room temperature. The slices were blocked with 3% bovine serum albumin (BSA)-phosphate buffer saline containing Tween-20 (PBST) for 90 min at room temperature and incubated with anti-GFAP antibody diluted 1/250 in PBST overnight at 4 °C in a humidified chamber. After washing 3–4 times with PBST, the slices were incubated for 60 min at room temperature with HRP-conjugated secondary antibody diluted 1/200 and rinsed thrice with PBST. The localization of GFAP on the slices was visualized using 0.05% 3.3′-diaminobenzidine and 0.03% peroxide hydrogen in 50 mM Tris buffer pH 7.4. The slices were then rinsed with distilled water, counterstained with Mayer hematoxylin, dry mounted with cresyl gel (0.1% w/v gelatine in 80% v/v ethanol) and coverslipped with Eukitt balsam. Immunoreactions were viewed with an Olympus CX41 light microscope (Olympus Europa SE & Co. KG, Hamburg, Germany). All procedures of the presented study were performed in the same conditions and in parallel. The figures were mounted with Adobe PhotoShop 7.0 software (San Jose, CA, USA).

Reactive oxygen species determination. Reactive oxygen species (ROS) in the fish brain was determined with the method described by Gupta et al. (2007) with a few modifications. The fresh isolated brain tissues taken individually from every fish was homogenized in Tris-HCl buffer (50 mM, pH = 7.4) on ice with ratio 1 : 10 w/v. The samples of brain tissue homogenate in volume 100 μL were mixed with 1 mL the same buffer and 5 μL of 10 μM 2′,7′-dichlorofluorescein diacetate (DCFDA). The obtained mixtures were incubated in 37 °C for 30 minutes (Lab. Companion SI-600 incubator shaker, Jelio Tech., Korea). After incubation, the fluorescence intensity was measured in the samples by using a fluorescence spectrophotometer LS55 (PerkinElmer, USA) with an excitation λ = 485 nm and an emission λ = 525 nm.

Statistical analysis. Data on the protein content are expressed as a percentage related to the control group and presented as a mean ± standard deviation (SD) in histograms. Quantitative results were analysed by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests. P values of $P < 0.05$ were considered to indicate statistical significance.

Results

The results of measuring GFAP in fish brain of the control group and those of the groups exposed to the copper sulphate showed inverse changes of the cytoskeleton protein that depend on both the duration and dose of the treatment. The data of GFAP relative content are presented in Figure 1. All the applied doses induced the downregulation of GFAP during the 72 hours treatment. In spite of the GFAP increment in common, surprisingly the exposure of 48 hours resulted in the upregulation of this astroglial marker. It should be noted that copper sulphate exposure induced several changes in β-actin content, especially in fish brain undergoing 72 hours treatment. The observed effect of copper in the fish brain may be related to the unspecific multiple disturbance and enlarged cytotoxicity in the brain tissue.

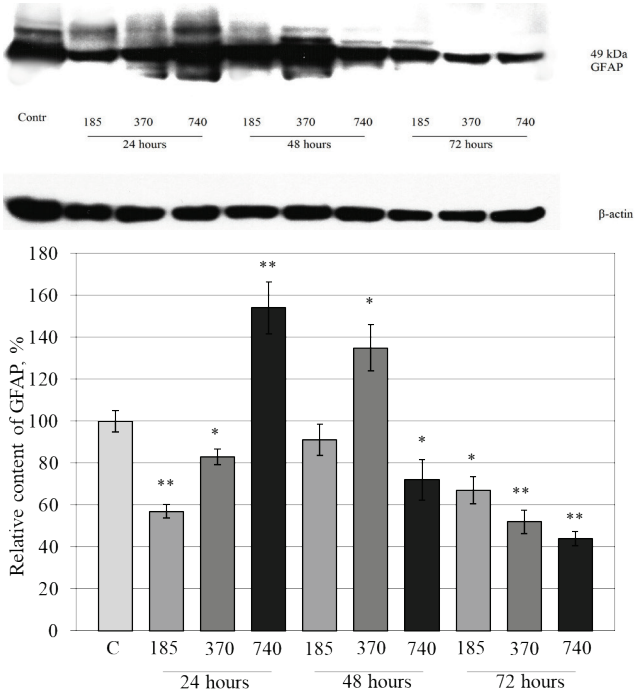

Fig. 1. Relative content of GFAP in fish brain (x ± SD, n = 68): C – control group; 185, 370, 740 – copper sulphate doses in μg/L; * – $P < 0.05$, ** – $P < 0.01$ vs. control

The observed effect of copper on the modulation of NF-kB content is presented in Figure 2. The discovered changes in the fish brain are evidence the dose-dependent effect in a concertation range 185–740 μg/L of copper sulphate in the groups exposed for 48 and 72 hours. By contrast, exposure to low dose of copper ions showed no effect in the fish group treated with copper sulphate for 24 hours.

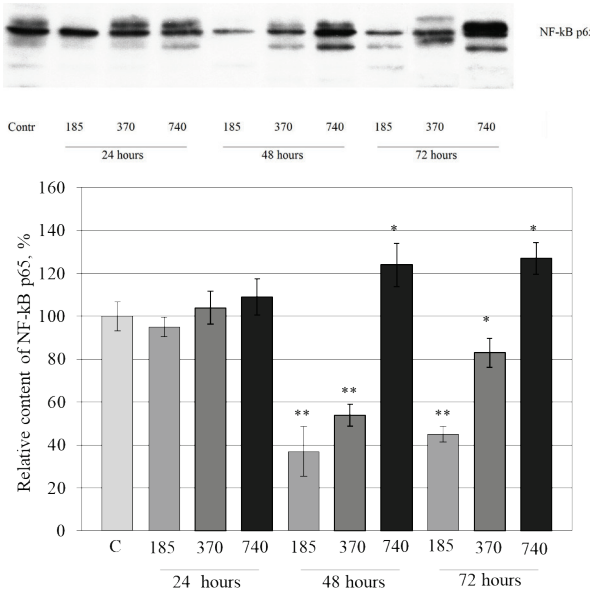

Fig. 2. Relative content of NF-kB in fish brain (x ± SD, n = 68): C – control group; 185, 370, 740 – copper sulphate doses in μg/L; * – P < 0.05, ** – P < 0.01 vs. control

The changes of PARP content in the brain were determined in each fish group differed by duration of treatment with copper sulphate (Fig. 3). The decrease of PARP content was observed in each group exposed to copper sulphate in a dose 740 μg/L. The exposure to 185 μg/L of copper sulphate did not induce significant changes in the PARP content in the fish brain in the group exposed for 24 hours. However, the treatment with 370 μg/L resulted in meaningful downregulation of the PARP content in the groups exposed to copper ions for 24 and 48 hours. Surprising results were obtained in all fish groups exposed to copper in a dose range 185–740 μg/L in the course of 72 hours. The downregulation of PARP content in fish brain from the group exposed to copper for 72 hours was significantly less than in the groups treated with copper for 24 and 48 hours. Thus, the obtained results support a supposition that copper ions in a dose range 185–740 μg/L can modulate the PARP expression in a time-dependent manner.

Taken together, the presented results provide vidence that sublethal doses of copper may induce in fish brain the complex deregulation of cytoskeleton and transcriptional regulators of the cellular response.

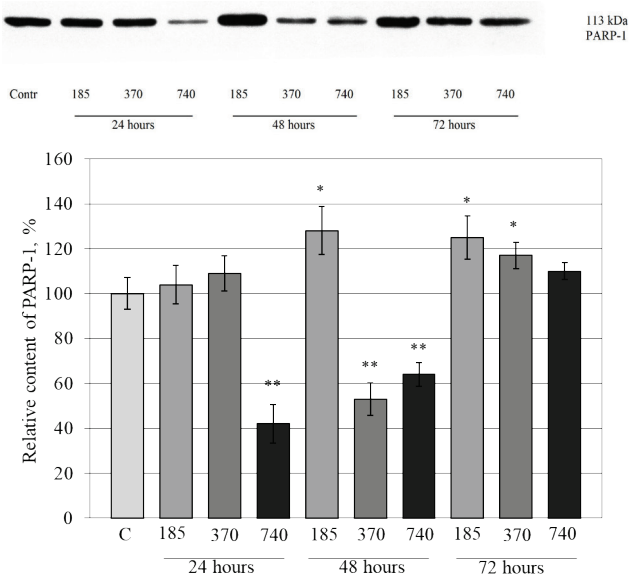

Fig. 3. Relative content of PARP-1 in fish brain (x ± SD, n = 68): C – control group; 185, 370, 740 – copper sulphate doses in μg/L; * – P < 0.05, ** – P < 0.01 vs. control

Immunohistochemistry of the brain slices was performed to determine copper-induced glial cell changes in the fish. The results of IHC show meaningful astroglial reactivity in fish brain induced by copper sulphate exposure to 740 μg/L dose at 72 hours (Fig. 4).

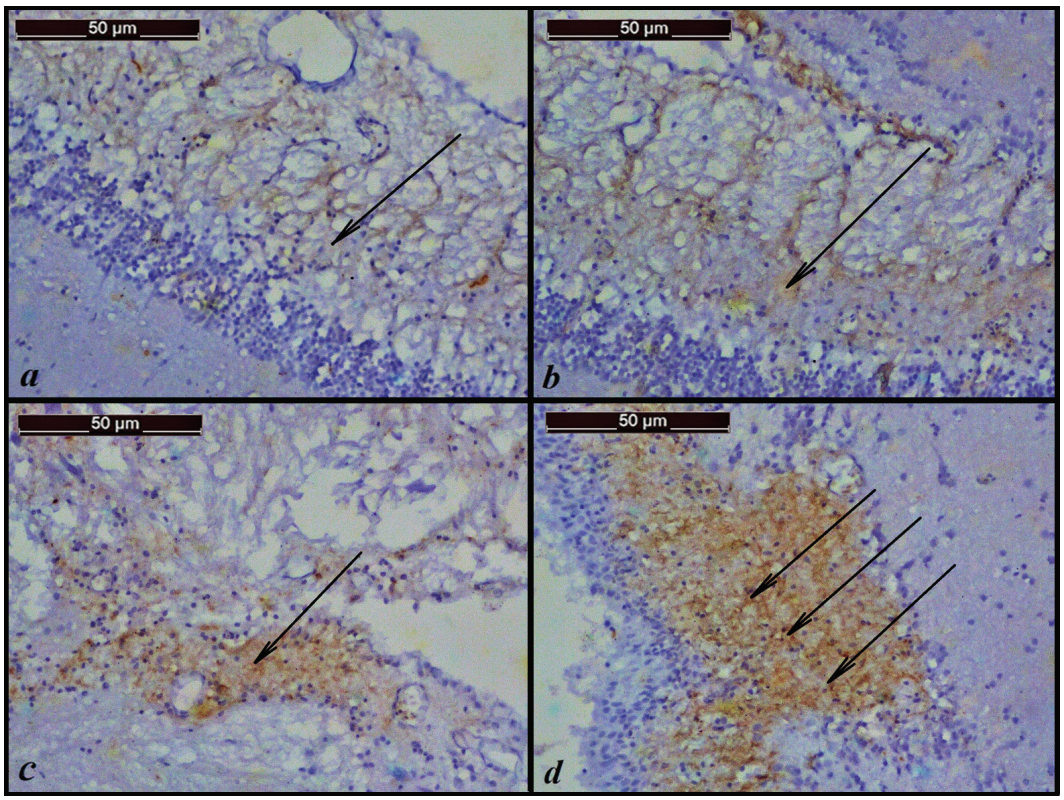

Fig. 4. Immunohistochemistry of the brain tissue of *Capoeta umbla* control (*a*) and exposed with copper sulphate: *b* – 185 μg/L, no visible effects; *c* – 370 μg/L, very slight traces of astrogliosis; *d* – 740 μg/L, severe developed astrogliosis

Immunohistochemistry results showed the copper sulphate (740 μg/L) treatment had induced local astrogliosis, which developed in some brain areas as it presented on Figure 4. Taking into account that metal toxicity is accompanied with oxidative stress, which is one of the essential causes that initiate the overactivation of cell response, we measured the ROS level to determine and validate this cause. Thus, in the presented study the brain ROS levels in fish of the control and exposed groups were determined to assess the copper ions as a cause of oxidative stress generation. The relative ROS content in all fish groups is presented in a Figure 5. The obtained data provide evidence of the pronounced time-dependent effect of copper in every fish group treated with the studied doses of copper sulphate.

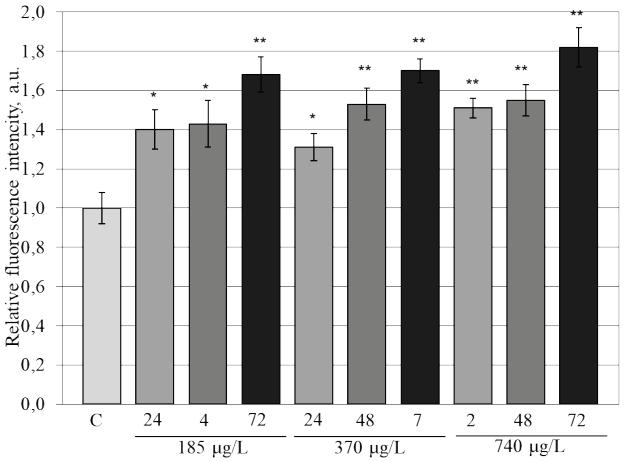

Fig. 5. Relative reactive oxygen species content in the fish brain (x ± SD, n = 68): C – control group; 185, 370, 740 – copper sulphate doses in μg/L; * – $P < 0.05$, ** – $P < 0.01$ vs. control; a.u. – arbitrary units

Discussion

Fish are recognized to be a relevant group for bioindication of heavy metals' pollution in aquatic ecosystems (Łuczyńska et al., 2018). However, researchers mostly study the heavy metals' content and oxidative stress indices in the gills, liver, gonads and muscles of fish. Extremely limited data on heavy metal cytotoxicity in fish brain are available.

Copper is an essential element of nutrition and the balance of copper level is critical for vital metabolic pathways (Olivares & Uauy, 1996). Physiological concentration of copper is regulated by the protein-binding and protein-transporting systems in tissues, especially in the liver, where it amounts 18–45 μg/g of the dry weight. Copper deficiency usually leads to seizures, and both blood and neural cell abnormalities in humans (Barceloux et al., 1999). Moreover, a lack of copper can cause pregnancy defects, especially brain development and cardiovascular disturbance that have been observed in many animal models (Madsen et al., 2007; Gitlin et al., 2007).

Copper is a transition metal and widespread inorganic ion in organisms of all vertebrate and invertebrate species. This ion is multifaceted and plays many roles in eukaryotic cells. In spite of its high significance, the excessive increment of copper level transforms this ion into a toxic agent. The ability of copper to exchange electrons easily makes it highly reactive, which results in cytotoxicity to eukaryotic cells and microorganisms. Despite that, copper is extremely important for key metabolic functions. Exposure to elevated content of copper ions can induce a number of disturbances in key pathways regulation. Copper is an essential element, but high concentrations develop cytotoxicity in many species of living organisms. Recently, Dornelles Zebral and colleagues presented the results of their research on copper hepatotoxicity. The authors proposed a physiological mechanism of the metal accumulation and accompanied elevated oxidative stress in the liver (Dornelles Zebral, 2019). Besides, they demonstrated evidence that copper can reduce antioxidant capacity and be critically hazardous to fish populations under increased temperature.

Taking into account the vulnerability of neural tissue cells to oxidative damages, our obtained data maintain a hypothesis on a close link between oxidative stress generation and glial cell response to copper neurotoxicity. Induced by copper exposure, the upregulation of GFAP expression as well as PARP and NF-kB activities could be parts of the cell complex response directed to survival of brain cells.

Besides, several researchers have presented experimental data that are evidence of the high toxic properties of copper for many exposed vertebrate species. Copper-induced cytotoxicity is closely associated with the disturbance of redox balance and increase in oxidative stress. Tseng and colleagues have reported that an exposure to Cu^{2+} resulted in the death of mouse liver cells, which was accompanied by increased nuclear condensation, DNA fragmentation and mitochondrial dysfunction (Tseng et al., 2012). Severe damage to the liver cells of both larval and adult zebrafish were demonstrated after exposure to a low 5 μM dose of Cu^{2+}. Moreover, the copper treatment reduced mitochondrial membrane potential, caspase-3 activation, and PARP cleavage that taken together may reflect an activation of apoptosis (Tseng et al., 2012). The liver is the main organ of xenobiotic detoxification as well as crucial target of copper cytotoxicity. It has been demonstrated that copper is intensively transported to the hepatic cells, which results in high metabolic activity of the liver tissue. Moreover, copper is accumulated in the liver due to the high affinity binding in the Cu-metallothionein complex in hepatocytes (Hogstrand & Haux, 1991; Perkins et al., 1997). The disturbance of liver function leads to a lack of detoxification and circulation of toxic metabolites in the blood. The brain cells are very sensitive to these toxic agents. Thus, copper toxicity could be realized through its direct effect on the brain cells as well as an indirect one mediated with hepatocyte damage.

Marcussen et al. (2008) showed that catfish (*Clarias gariepinus*) could accumulate Cu^{2+} from the aquatic environment contaminated with $CuSO_4$. Therefore, copper bioaccumulation in the liver was determined as much more intensive in comparison with gills, muscle, and skin. Unfortunately, there are no available data on copper accumulation in fish brain. Moreover, the toxic effects of Cu^{2+} in neural tissue cells are still unstudied. In addition, the mechanisms of $CuSO_4$ cytotoxicity in neural tissue are unidentified and require integrative research of cell and molecular response to Cu^{2+} exposure (Deshpande et al., 2013; Brander et al., 2016). Tan et al. (2008) determined the differences in cytotoxic effects of four heavy metals: cadmium (Cd), chromium (Cr), zinc (Zn), and copper (Cu) for several fish species. Comparative analysis of these transition metals showed that most cytotoxic effect was developed by copper in kidney cells of the grass carp (*Ctenopharyngodon idellus*).

Cytotoxic concentrations of copper can induce disturbances that lead to oxidative modifications of DNA bases and to breaks of DNA strand. Guecheva et al. (2001) showed that copper ions inhibit the repair of DNA damage induced by methyl methanesulphonate. Thus, environmental sources of copper exposure can modulate the genotoxic effect mediated by other environmental pollutants.

One of the most studied mechanisms of DNA repair in eukaryotic cells is the synthesis of poly-ribose by the universal enzyme named poly-[ADP-ribose]-polymerase 1 (PARP-1) (Gibson & Kraus, 2012). Kim et al. (2014) showed the genotoxic effect of polycyclic aromatic hydrocarbons, a group of highly toxic xenobiotics, both in cell lines and zebrafish. Observed genotoxicity was associated with notable deregulation of PARP-1. Notably meaningful PARP inhibition was observed in embryos. The results obtained in our study confirmed significant deregulation of PARP-1 in the fish brain because of exposure to copper ions. The disturbance of PARP-1 expression may induce changes of ADP-ribosylation and poly-ribose blend that are responsible for DNA repair. ADP-ribosylation is one of the key regulators of cellular processes, including the regulation of dynamic rearrangement of chromatin, transcription, protein translation, and conformational stability of the end products (Hottiger, 2015). We found pronounced deregulation of PARP-1 expression in the brain of *C. umbla* exposed to copper in a range of doses 185–720 μg/L. Thus, the toxic effects of copper can be related to PARP-1 deregulation as was demonstrated in our study of the Tigris scraper.

The liver and gills are the most frequently used fish tissue as an object to study of the toxic effects of various environmental pollutants.

Copper induces intracellular damage, apoptosis, inhibits the antioxidant systems, and breaks intercellular adhesion. Wang and co-authors demonstrated that treatment of young grass carp (*Ctenopharyngodon idella*) with 11 μM copper disrupts an expression of tight junction proteins in the fish gills (Wang et al., 2015). The same changes were determined in the liver of channel catfish following a 10-week exposure to copper sulphate (Perkins et al., 1997). Thus, the copper exposure may induce complex pathogenesis in different tissues of fish.

In the presented study, we have investigated the content of soluble GFAP in the brain of *C. umbla*. The content of this GFAP form is regulated by different mechanisms in glial cells as well as the insoluble GFAP form (Nedzvetsky et al., 2006). The increase of the soluble GFAP form may be modulated by both transcriptional activity of GFAP genes and the local proteolysis of glial intermediate filaments presented in cells. As this takes place, the decrease of the soluble GFAP form depends on the inhibition of intracellular proteases and expression of several isoforms of glial intermediate filament protein. The obtained results provide evidence of the presence in fish brain cells of a complex mechanism of the influence of different copper doses on GFAP regulation. Linear dose-dependent effect of copper exposure was observed in the fish groups treated during 24 and 72 h. Despite the nonlinear nature of the dose-dependent effect of copper exposure in fish treated during 48 hours, the results obtained in our study suggest that both the dose and the duration of copper exposure are crucial for cell response and cell cytoskeleton disorders.

GFAP expression is the most commonly used biomarker of the astroglial reactivity (Eng et al., 2000). The GFAP content is implicated in the structure and function of the cell cytoskeleton (Yang & Wang, 2015). The response of astrocytes to many CNS injuries is named a reactive gliosis or astrogliosis. Many studies have shown that a modulation of GFAP expression alters the capability of astrocytes to proliferate and other vital features (Pekny et al., 2014; Bramanti et al., 2016; Nedzvetskii et al., 2016). Astrocytes are the most abundant cell population in the brain of vertebrates. Astroglial cells provide the protection against excitotoxic damage and maintain both the structure and the functions of neurons. The reactivation of astrocytes is one of the main events in defence reactions against various damage factors.

In our investigation, we report that copper modulates GFAP expression in the fish brain. Different durations of copper exposure induced reciprocal changes, in particular low and high doses stimulated more intensive downregulation of GFAP than the middle dose. These results may reflect the dose-dependent dynamic of astrocyte reactivity as a response to copper cytotoxicity. Thus, the protective effect of astrogliosis is developed as a nonlinear function and depends on intensity of the stimulus and its duration as well.

The mechanisms of the regulation of GFAP expression in the fish brain and dose-dependent astrocyte reactivity remain unknown. The modulation of GFAP content in astrocytes with copper exposure depends on both the expression activity and the intensity of cleaving by proteases. A large amount of data supports the evidence that GFAP and its proteolytic fragments are promising molecular biomarkers for different CNS pathogenic disorders. It was demonstrated that the fragmentation of GFAP is generated by calpaines and caspases (Chen et al., 2013). Moreover, the fragments of glial intermediate filaments resulting from the proteolysis are also able to initiate filament aggregation and to develop the proapoptotic propriety. On the other hand, it was shown that copper exhibits dose-dependent proapoptotic effect in several cancer cell lines. Thus, copper ions can stimulate GFAP overexpression in astrocytes as well as induce GFAP fragmentation which should entail a decrease in GFAP content.

The results obtained in our study accord with the data presented by Acarin et al. (2007). They demonstrated that excitotoxic damage in the brain of postnatal rats is accompanied by GFAP overproduction and glial scar formation. The changes observed in the study were accompanied with a rise in caspase-3 enzymatic activity that could initiate an increment of caspase-cleaved fragments of GFAP.

In the brain of vertebrates, GFAP overexpression is associated with upregulation of the proinflammatory excretion of factors. In our experiments, we observed the modulation of NF-kB expression, which is accompanied by GFAP upregulation in fishes from the group exposed to copper. The mechanisms of NF-kB activation are multifaceted. F-kB activation in astroglial cells may be initiated by various stimuli, including overproduction of ROS and reactivation of cells (Morgan & Liu, 2011). In light of these observations, it is natural to hypothesize that copper-induced astrocyte reactivation in the fish brain is regulated by NF-kB-dependent pathways. One of them may control the cytoskeleton rearrangement and the cell response to toxic effects of environmental pollutants. Moreover, astroglial reactivation in the fish brain was accompanied by a modulation of both key regulator of transcriptional activity: NF-kB (Fig. 1, 2) and DNA-repairing enzyme PARP. The obtained results demonstrate the dose-dependent effects of copper on neural tissue cells' response to toxic exposure.

Recent achievement in transcriptome sequencing demonstrated a wide prevalence of NF-kB transcriptional activity in many vertebrates and invertebrates, which should be evidence of the universal role of that regulation (Gilmore et al., 2012; Wolenski et al., 2012). The study of the role of NF-kB in cellular response regulation in the fish brain could be useful in clarifying signalling pathway switching to neural tissue cell survival. Moreover, the elucidation of transcriptional regulation in brain tissue cells may give us the information on the range (frontiers) of cell reactivation induced by extremely toxic environmental pollution.

The regulation of cell response to the influence toxic xenobiotics requires the sharp balance in activation-inhibition of the NF-kB system (Cao et al., 2017). Excessive activation of the NF-kB may induce the disturbance in transcriptional level of the proinflammatory factors which results in its leaking and finally cell death. However, moderate activation facilittes cell survival and directs the pathways to detoxification. NF-kB activation is closely related to oxidative stress. The growth of free radicals is one of the usual factors that induce NF-kB-dependent cell response. The role of antioxidants in the regulation of NF-kB p65 activity in juvenile grass carp was demonstrated recently (Zeng et al., 2016). Moreover, it was shown that the oxidative stress induced by excessive copper content may be prevented by NF-kB activation in human embryonic kidney and mouse embryonic fibroblast cell cultures (Kenneth et al., 2014).

Doherty et al. (2016) presented the results of histopathology studies of the gills as a good biomarker for detecting pesticide toxicity in fish. The histopathological changes were associated with a reduction of acetylcholinesterase level in the fish exposed to pesticides. In the presented study, we used the immunohistochemistry (IHC) to detect abnormalities in the fish brain. The results of IHC staining with anti-GFAP antibody revealed astrogliosis in the brain of fishes exposed to copper sulphate. The observed changes reflect the response of astrocytes to copper toxicity and could be one of the key parts of cell reactivity directed to providing neural tissue function.

Several studies demonstrated the prooxidant effect of copper in some fish tissues (Tseng et al., 2012; Wang et al., 2015). However, in our research we determined the time-dependent effect in *C. umbla* brain, which supports the hypothesis of the cumulative toxic effect of copper ions. Neural tissue has a low antioxidant potency as well as more intensive oxygen consumption as compared to other tissues. Thus, ROS-induced damage in neural tissue may be built up during exposure to toxins, especially to copper ions.

The reactivation of glial cells observed in our study is accompanied by a modulation of NF-kB and PARP-1 expression. The obtained results can reflect a functional relation between glial cytoskeleton rearrangement and an activation cell response to copper toxicity. Moreover, the absence of meaningful dose-dependent effect of sublethal doses of copper could be caused by the multiple feedbacks between molecular targets of both NF-kB and PARP-1. Taking into account that PARP-1 can trigger cells to apoptosis, different doses of copper ions can disturb the balance in programmed cell death regulation.

Repairing DNA damage is well known to be one of the key functions of PARP. However, there are numerous data that PARP can participate in the modulation of brain tissue cell death initiated by different disorders (Moroni, 2008) or glutamate induced insults (Meli, 2004). Overactivation of PARP in the course of cell response is accompanied by the excessive consumption of its substrates, especially NAD^+ and, as

a consequence, the depletion of the ATP level. Prolonged and excessive PARP activation induces the metabolic energy deficit that provokes the switching of signalling pathways to cell death.

Taking into account that PARP takes part in the cellular response as a NF-kB coactivator in translational regulation of proinlflammatory factors, PARP activity can modulate the expression of proteins involved in the inflammatory cascade process as well as in the universal kinase pathways that stimulate the cell reactivity of positive feedback mechanisms. Thus, the nonlinear dependence in GFAP, PARP and NF-kB expression observed in our study could be the superposition of multiple pathways, which modulate cellular response against Cu toxicity.

The present study was performed with the use of different sublethal concentrations of Cu^{2+} that induce stress response in the fish brain. However, it is still unknown how many cells can die later in a consequence of this treatment. Thus, the study of copper toxicity requires relevant proceedings that make a special point of the response in brain cell cultures.

Conclusion

Taking into consideration that GFAP upregulation is a biomarker of various neurotoxicity types, the astroglial reactivity observed with IHC and Western blot in the fish brain is evidence of the toxic effect of copper ions in the cells of neural tissue. The differential changes of GFAP, NF-kB, and PARP expression in the fish brain presented in our study may reflect the complex reactivation induced by copper ions in the neural tissue cells of the Tigris scraper. The sublethal doses of copper ions can provoke the total deregulation of crucial pathways that control cellular response against heavy metal ions' toxicity. NF-kB-dependent pathways are essential in both cell reactivation and cellular vital capacity.

Our results indicate that the activation of NF-kB, PARP as well as astrogliosis in the fish brain may be important for animal survival in environments with elevated concentrations of copper.

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