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Acute exposure to the penconazole-containing fungicide Topas partially augments antioxidant potential in goldfish tissues

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ABSTRACT

Penconazole is a systemic fungicide commonly used in agriculture as the commercial preparation Topas. Although triazole fungicides are widely found in the aquatic environment, little is known about their acute toxicity on fish. In this study we assessed the effects of short-term exposure to Topas on some parameters of homeostasis of reactive oxygen species (ROS), such as the levels of markers of oxidative stress and parameters of the antioxidant defense system of goldfish (*Carassius auratus* L.). Gills appeared to be the main target organ of Topas toxicity, showing the greatest number of parameters affected. Gills of Topas-treated fish showed a higher content of low (L-SH) and high (H-SH) molecular mass thiols and higher activities of superoxide dismutase (SOD), catalase, glutathione reductase (GR), glutathione-*S*-transferase (GST), and glucose-6-phosphate dehydrogenase (G6PDH) as well as reduced carbonyl protein content (CP), as compared with those in the control group. In the liver, goldfish exposure to 15–25 mg L^{-1} Topas resulted in a higher L-SH and H-SH content, but lower CP levels and activity of GST. In kidney, Topas exposure resulted in higher activities of glutathione peroxidase (GPx) and G6PDH, but lower L-SH content and activity of GST. The results of this study indicate that acute goldfish exposure to the triazole fungicide Topas increased efficiency of the antioxidant system in fish gills, liver, and kidney. This could indicate the development of low intensity oxidative stress which up-regulates defense mechanisms responsible for protection of goldfish against deleterious ROS effects.

1. Introduction

Penconazole [1-(2,4-dichloro-β-propylphenethyl)-1H-1,2,4-triazole] belongs to the group of systemic triazole fungicides commonly used in the horticultural, agricultural, and forestry industries for foliar pathogen control (Pose-Juan et al., 2010). It is an active ingredient of many fungicides that are used worldwide such as Topas, Omnex, Oron, Ofir and Dallas. Fungicidal properties are achieved via inhibition of the cytochrome P450-dependent 14α-demethylase activity required in the conversion of lanosterol to ergosterol, an essential component of fungal biological membranes, and disintegration of cellular membranes (Henry and Sisler, 1984; Roberts and Hutson, 1999). At high levels, members of this class of fungicides have a variety of toxicological outcomes in mammals including carcinogenicity, reproductive toxicity, and hepatotoxicity (Zarn et al., 2003; Juberg et al., 2006; Peffer et al., 2007).

These fungicides are normally sprayed directly on plants and are rapidly absorbed and distributed to the interior of the leaves. However, some fraction of the applied pesticides can reach the soil due to drifting during application, rain washing the pesticides off the foliage, and plant material falling onto the soil. Therefore, triazole fungicides constitute a risk to soil ecosystems as well as to ground and surface waters (Kim et al., 2002; Castillo et al., 2006; Kahle et al., 2008; Komárek et al., 2010; Zheng et al., 2016). Penconazole residues have been found in vineyard soils of the Galician province Pontevedra (Northwestern Spain) at a concentration of 50 μg kg^{-1} (Arias et al., 2006). In the Spanish province Ourense, penconazole was also found in soils sampled throughout the year (up to 411 μg kg^{-1}), with the higher concentrations observed in summer and spring during the applications (Bermúdez-Couso et al., 2007). Nevertheless, little is known about penconazole availability in surface water. Indeed, this fungicide was found in surface waters in the Western Cape (South Africa) in concentrations below 2 μg L^{-1} (Dalvie et al., 2003).

In freshwater ecosystems, penconazole can generate adverse effects to aquatic organisms, such as fish. They can accumulate different triazoles rapidly during the 8 days uptake phase, and followed by

Abbreviations: CP, carbonyl protein groups; GPx, glutathione peroxidase; GST, glutathione-*S*-transferase; GR, glutathione reductase; G6PDH, glucose-6-phosphate dehydrogenase; H-SH, high molecular mass thiols; LOOH, lipid peroxides; L-SH, low molecular mass thiols; ROS, reactive oxygen species; SOD, superoxide dismutase.

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rapid elimination (Konwick et al., 2006). Penconazole LC_{50}^{96} values for rainbow trout, channel catfish, bluegill sunfish and carp were found to be in the range 1.3–4.6 mg L^{-1} (Surprenant, 1984a, 1984b, 1984c; Rufli, 1984). However, knowledge of the effects of commercial formulations of triazole fungicides on fish is quite limited. Triazoles were found to be potent effectors of cytochrome P_{450} enzymes in rainbow trout (*Oncorhynchus mykiss*) (Levine et al., 1999; Hinfray et al., 2006). Several triazole fungicides depress circulating sex steroid concentrations, thus reducing egg production (Liao et al., 2014; Skolness et al., 2013). Long- and short-term exposure to these fungicides affect both morphological indices and other biochemical parameters in fish, including antioxidant responses, hematological changes, and RNA/DNA ratio (Li et al., 2010a; Li et al., 2013; Zhu et al., 2014).

Therefore, the present study was undertaken to investigate the effects of the industrial penconazole-containing fungicide Topas 100 EC on free radical processes in the gills, liver, and kidney of goldfish (*Carassius auratus* L.).

2. Material and methods

2.1. Reagents

Phenylmethylsulfonyl fluoride (PMSF), 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), oxidized glutathione (GSSG), β-nicotinamide adenine dinucleotide phosphate (NADP), β-nicotinamide adenine dinucleotide reduced (NADH), glucose-6-phosphate (G6P), ethylenediamine-tetraacetic acid (EDTA), xylenol orange, cumene hydroperoxide, ferrous sulphate, 2,4-dinitrophenylhydrazine (DNPH), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), hydrogen peroxide (H_2O_2), NaCl, KH_2PO_4, Tris (hydroxymethylaminomethane), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), pyruvic acid, glutathione reductase from baker's yeast, and β-nicotinamide adenine dinucleotide phosphate reduced (NADPH) were purchased from Sigma–Aldrich Corporation (USA). Topas 100 EC was purchased from Syngenta Crop Protection AG (Switzerland). All other reagents were of analytical grade.

2.2. Animals and experimental conditions

Goldfish (*C. auratus* L.), with body mass of 80–100 g, were obtained from a local fish farm (Halych district, Ivano-Frankivsk region, Ukraine) in October 2014. Fish were acclimated to laboratory conditions for four weeks in a 1000 L tank under natural photoperiod in aerated and dechlorinated tap water. Water parameters were 20 ± 2 °C, pH 7.6–7.8, 7.0–7.6 mg L^{-1} O_2 and hardness (determined as Ca^{2+} concentration) of 39–40 mg L^{-1}. Fish were fed ad libitum with commercial pellets for pond fish "Tetra Pond Sticks" (Tetra, Germany), containing 28% protein, 3.5% fat and 2% cellulose, Zn (48 mg kg^{-1}), Fe (31 mg kg^{-1}), Mn (81 mg kg^{-1}), Co (0.6 mg kg^{-1}), and vitamins A and D_3. Fish were fed during the acclimation period (4 weeks), but were fasted for one day prior and during experimentation.

Experiments were carried out in 120 L glass aquaria (containing 100 L of water), in a static mode, under the same conditions, but with the addition of the commercial fungicide Topas (Syngenta Crop Protection AG, Switzerland) which contains penconazole [1-(2,4-dichloro-β-propylphenethyl)-1H-1,2,4-triazole] at a concentration of 100 g L^{-1}. Groups of five fish were placed in aquaria with different nominal concentrations of Topas fungicide: 1.5, 15 or 25 mg L^{-1}. Topas concentrations used in this work were selected based on an LC_{50}^{96} value (half-lethal concentration after 96 h exposure) for penconazole exposure of carp determined to be 3.8–4.6 mg L^{-1} (Rufli, 1984). Fish were exposed to these conditions for 96 h (no mortality occurred during exposures). Fish in a control group were maintained in the same manner, but without addition of Topas to the water. Aquarium water was not changed over the 96 h course in order to avoid stressing the animals. Levels of dissolved oxygen, temperature and pH were monitored every 24 h. The experiments were carried out in two independent experimental replicates with a total number of at least five biological replicates for every measured parameter.

After exposure, fish were sacrificed by transspinal transsection without anesthesia and tissues (gills, liver, and kidney) were dissected, rinsed in ice-cold 0.9% NaCl, dried by blotting on filter paper, frozen, and stored at −70 °C until use. All experiments were conducted in a strict accordance with the Ethics Committee of Precarpathian National University.

2.3. Determination of oxidative stress indices

Lipid peroxide (LOOH) content was assayed by the FOX (ferrous-xylenol orange) method (Hermes-Lima et al., 1995). Tissue samples were homogenized (1:5, w:v) using a Potter–Elvehjem glass homogenizer in 96% ethanol (4 °C) and centrifuged (5000*g*, 15 min, 4 °C). Aliquots of the supernatants were used for the assay as described previously (Lushchak et al., 2005). The content of LOOH was expressed as nanomoles of cumene hydroperoxide equivalents per gram wet mass of tissue.

Carbonyl groups of proteins (CP) in tissues were determined as described previously (Lushchak et al., 2005). Tissue samples were homogenized (1:10, w:v) in homogenization medium (50 mM potassium phosphate buffer, pH 7.0, 0.5 mM EDTA, 1 mM PMSF) and centrifuged (16,000*g*, 15 min, 4 °C). Supernatants were removed and 0.25 mL aliquots were mixed with 0.25 mL of 40% trichloroacetic acid (TCA) (final TCA concentration 20%) and centrifuged (5000*g*, 5 min, 21 °C). CP levels were measured in the resulting pellets by reaction with 2,4-dinitrophenylhydrazine (DNPH), leading to formation of dinitrophenylhydrazones (Lenz et al., 1989). Values are expressed as nanomoles of carbonyl groups per milligram protein (nmol mg $protein^{-1}$).

Free thiols were measured spectrophotometrically by the Ellman procedure with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) at 412 nm (Ellman, 1959). Total thiol concentration (the sum of low and high molecular mass thiols) was measured in supernatants prepared as for the CP assays as described previously (Lushchak and Bagnyukova, 2006). For determination of low-molecular mass thiols (L-SH), aliquots of supernatants were mixed with TCA to reach a final TCA concentration of 10%, centrifuged (16,000*g*, 5 min, 21 °C) to remove pelleted protein and the final supernatants were used for the assay. Thiol concentrations were expressed as micromoles of SH-groups per gram tissue wet mass. The high-molecular mass thiol (H-SH) content was calculated by subtracting the L-SH concentration from total thiol concentration.

2.4. Assay of enzyme activities and protein concentration

Tissue supernatants were prepared as described above for the CP/thiol assays. The activities of antioxidant enzymes including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) as well as the activities of antioxidant-associated enzymes glutathione-*S*-transferase (GST), glutathione reductase (GR), and glucose-6-phosphate dehydrogenase (G6PDH) were measured as described previously (Lushchak et al., 2005).

One unit of SOD activity was defined as the amount of enzyme (per mg protein) that inhibited a quercetin oxidation reaction by 50% of maximal inhibition. Inhibition values for SOD activity were calculated using an enzyme Kinetics computer program (Brooks, 1992). One unit (U) of catalase, GST, GR, GPx, or G6PDH activity is defined as the amount of enzyme that consumed 1 μmol of substrate or generated 1 μmol of product per minute. Activities were expressed as international units (or milliunits) per milligram soluble protein (U mg $protein^{-1}$ or mU mg $protein^{-1}$).

Soluble protein concentrations were measured by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as a standard.

2.5. Statistics

Data are presented as means ± S.E.M. Statistical analysis was performed using analysis of variance ANOVA followed by the Dunnett's test to compare multiple experimental treatments to the single control value. The probability value of $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

It is known that environmental pollution can enhance the production of ROS, such as superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^{\bullet}$) (Fatima et al., 2007; Slaninova et al., 2009; Lushchak, 2011). Several toxicological studies have confirmed that acute or chronic exposure to triazole fungicides enhanced ROS generation and induced oxidative stress in fish (Li et al., 2010a; Zhu et al., 2014; Tu et al., 2016). However, there is a scarcity of data on the effects of acute and chronic exposure to penconazole or its commercial formulations at sublethal concentrations. Therefore, we decided to fill this knowledge gap with an evaluation of the effects of acute exposure to the penconazole-based fungicide Topas on oxidative stress markers and antioxidant enzymes in the goldfish tissues. We chose three tissues (gills, liver, kidney) to evaluate ROS-related parameters under normal conditions and at fungicide exposure. Gills are primary organs that are directly exposed to water-borne contaminants whereas liver and kidney are key organs involved in elimination, biotransformation, and excretion of xenobiotics from the body.

3.1. Levels of ROS-modified cellular components in Topas exposed goldfish

The concentration of CP groups in tissues of control goldfish were highest in gills (10.6 ± 1.5 nmol mg protein^{-1}), intermediate in liver (2.61 ± 0.29 nmol mg protein^{-1}), and lowest in kidney (1.74 ± 0.50 nmol mg protein^{-1}). Fish exposure for 96 h to 1.5–25 mg L^{-1} Topas significantly decreased CP levels in gills by 26–40%, but did not affect this parameter in kidney (Fig. 1A). In liver, CP levels were lower by 43% in fish exposed to 25 mg L^{-1} of Topas than that in the control group (Fig. 1A). The lower CP levels in Topas-treated animals could be related to either enhanced proteolytic degradation of ROS-modified proteins, or stimulated synthesis de novo of cell proteins which could result in a "dilution" of damaged proteins. Additionally, selective elimination of ROS-modified proteins could take place due to disintegration of gills and liver cells. In contrast to our results, Li and colleagues observed increased levels of CP in gills and no effect in liver of rainbow trout (*Oncorhynchus mykiss*) after acute (96 h) exposure to another triazole fungicide, propiconazole (Li et al., 2013). Also, the level of CP was increased in liver of *Rhamdia quelen* exposed to tebuconazole for 96 h (Ferreira et al., 2010) and a dose dependent increase in CP levels was demonstrated in liver of medaka fish larvae exposed to propiconazole for 14–28 days (Tu et al., 2016).

In control goldfish, the concentration of lipid peroxides (LOOH) was highest in liver (689 ± 47 nmol gwm^{-1}), intermediate in kidney (110 ± 13 nmol gwm^{-1}), and lowest in gills (49.6 ± 4.4 nmol gwm^{-1}) (Fig. 1B). None of the tissues showed statistically significant changes in LOOH concentration after goldfish exposure to Topas at any concentration used, suggesting that oxidative modification of lipids didn't occur. Similarly, acute exposure to propiconazole at a concentration of 5.04 mg L^{-1} had no effect on the intensity of lipid peroxidation, measured as levels of thiobarbituric acid reactive substances (TBARS), in liver of juvenile rainbow trout, but significantly higher levels of TBARS were observed in the gills (Li et al., 2013). In contrast to our results, the TBARS levels were significantly increased in common carp (*Cyprinus carpio*) after acute exposure (96 h) to triazole fungicide, tebuconazole (Toni et al., 2011). Tu et al. (2016) reported elevated level of the lipid peroxidation product malondialdehyde (MDA) in liver of medaka fish after long term (14–28 days) exposure to propiconazole.

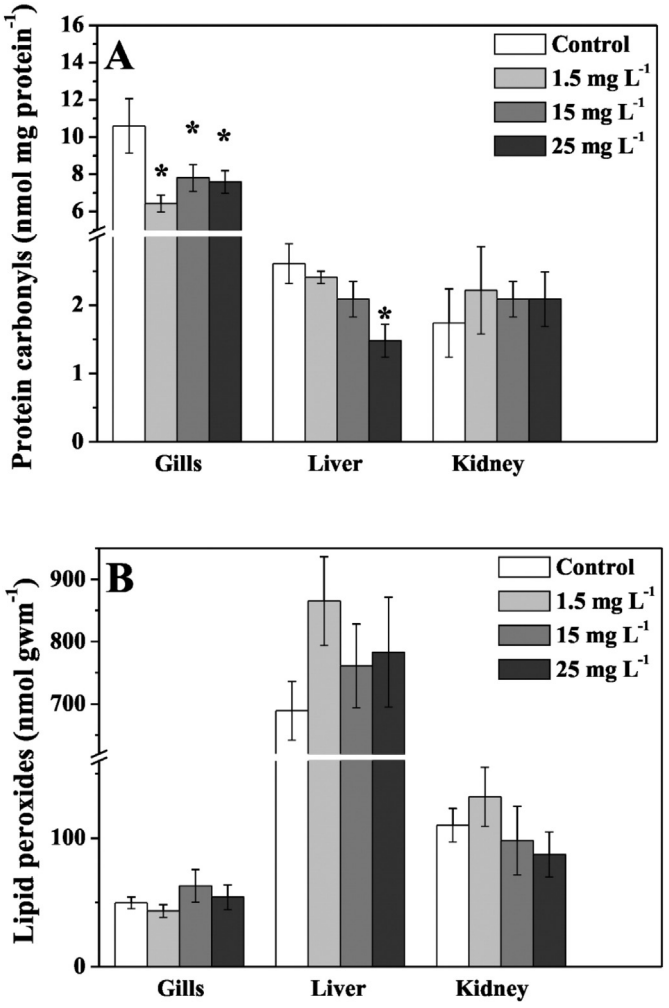

Fig. 1. The level of carbonyl proteins (nmol mg protein^{-1}) (A) and lipid peroxides (nmol gwm^{-1}) (B) in gills, liver and kidney of goldfish, exposed to control conditions or 1.5, 15, and 25 mg L^{-1} of Topas for 96 h. Data are presented as means ± S.E.M, n = 7–10. *Significantly different from the control group ($P < 0.05$) using ANOVA followed by a Dunnett's test.

The levels of intracellular thiol-containing compounds can also be affected by ROS (Lushchak and Bagnyukova, 2006). In this study, control levels of high-molecular mass thiols (H-SH; representing the sulfhydryl groups in proteins) were lowest in liver (2.62 ± 0.15 μmol gwm^{-1}), intermediate in gills (4.69 ± 0.24 μmol gwm^{-1}), and highest in kidney (5.77 ± 0.16 μmol gwm^{-1}) of control goldfish (Fig. 2A). Fish exposure to Topas had no effect on H-SH content in kidney, whereas the exposure to 15 and 25 mg L^{-1} of fungicide caused enhanced levels of H-SH in gills by 18 and 19%, respectively, as compared with the control group (Fig. 2A). In liver, the H-SH content was higher by 34% in fish exposed to 25 mg L^{-1} Topas than that in the control group (Fig. 2A). Low molecular mass thiols (L-SH) are represented by cysteine, homocysteine and the tripeptide glutathione (GSH) (Lushchak, 2012). Glutathione is commonly the most abundant low molecular mass thiol in animal cells. GSH-related processes play a central role in the second line of antioxidant defense by contributing to a number of processes, such as free-radical scavenging, reduction of peroxides, and detoxification of electrophilic compounds (Maher, 2005; Lushchak, 2012). GSH is involved in detoxification of pesticides via conjugating with them either nonenzymatically or with the participation of GSH-transferases. Moreover, glutathione is used as a substrate by GSH-related enzymes, including GST and GPx, in processes of detoxification of lipid peroxides (Lushchak, 2012). In the present study, the concentration of L-SH in

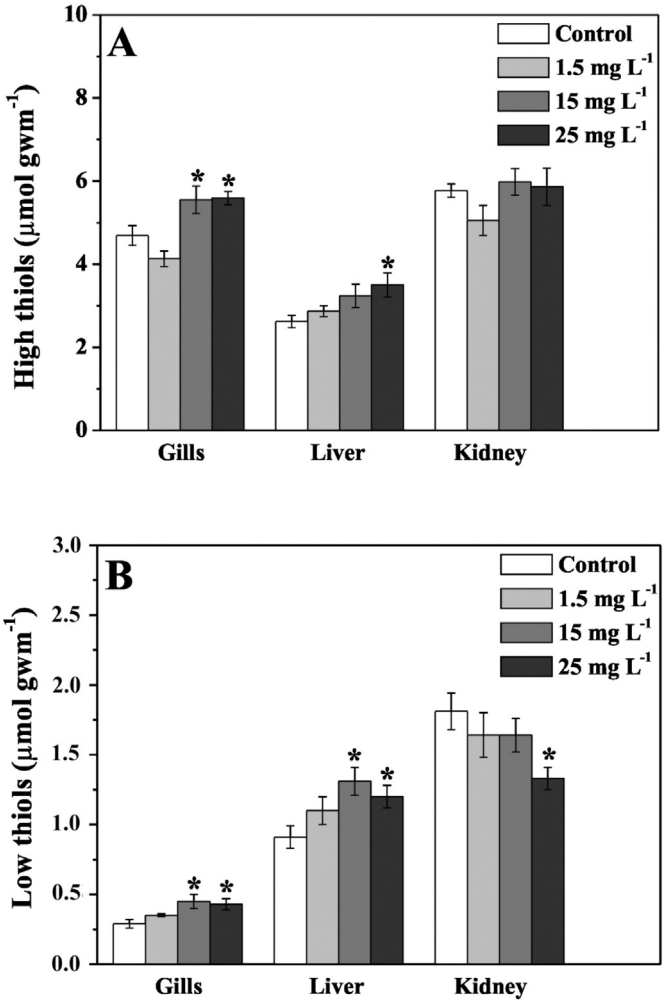

Fig. 2. The concentrations of high molecular mass (A) and low molecular mass (B) thiols (μmol gwm^{-1}) in gills, liver and kidney of goldfish, exposed to control conditions or 1.5, 15, and 25 mg L^{-1} of Topas for 96 h. Other information as in Fig. 1.

control fish was lowest in gills (0.29 ± 0.03 μmol gwm^{-1}), intermediate in liver (0.91 ± 0.08 μmol gwm^{-1}), and highest in kidney (1.81 ± 0.13 μmol gwm^{-1}) (Fig. 2B). Fish exposure to 15 and 25 mg L^{-1} Topas led to an increased concentration of L-SH by 55 and 48% in gills and 44 and 32% in liver, respectively, as compared to the control values (Fig. 2B). Such effects could result either from increased thiols biosynthesis, or decreased its consumption and/or degradation. Previously, we also found increased L-SH concentrations in gills of goldfish treated with the mancozeb-containing carbamate fungicide Tattoo (Kubrak et al., 2012). However, in kidney of goldfish exposed to 25 mg L^{-1} Topas the concentration of L-SH was reduced by 73% as compared with control values (Fig. 2B). Kidney is a target organ for certain toxicants since it actively transforms xenobiotics and is a major route for their excretion. Thus, the concentration of L-SH may decrease in kidney due to their conjugation with ROS and xenobiotics. A decrease in the concentration of L-SH also might result from utilization of GSH as electron donors in the enzymatic reactions (Sakhi et al., 2006). Li et al. (2010b) reported decreased GSH levels in gills and liver of rainbow trout after long term exposure (20 and 30 days) to propiconazole. The GSH content in liver of *Sebastiscus marmoratus* was also significantly reduced in a dose-dependent manner after 50-day exposure to another triazole fungicide, paclobutrazol (Li et al., 2012). However, the non-protein thiol content was increased (42% as compared to control fish) in liver of *R. quelen* exposed to tebuconazole (Ferreira et al., 2010).

3.2. Effect of Topas on antioxidant enzyme activities

The effects of Topas exposure on the activities of antioxidant enzymes were also assessed in the three goldfish tissues. Superoxide dismutase (SOD) and catalase have related functions, and are considered to be the first line of defense against oxygen toxicity, due to their inhibitory effects on oxyradical formation (Van der Oost et al., 2003).

Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide anion radical to hydrogen peroxide and molecular oxygen (Nordberg and Arner, 2001). The activity of SOD in control goldfish was highest in liver (275 ± 26 U mg $protein^{-1}$) and lower in kidney and gills (97.1 ± 10.6 and 44.4 ± 4.1 U mg $protein^{-1}$, respectively) (Fig. 3A). Fish exposure to Topas did not affect SOD activity in either liver or kidney. In gills, a significant enhancement of SOD activity by 41 and 37% occurred in fish treated with 15 and 25 mg L^{-1} Topas (Fig. 3A). Such an increase could be due to an up-regulation of SOD protein content in response to enhanced $O_2^{\bullet -}$ levels which can be considered as an adaptation of goldfish to overcome excess production of $O_2^{\bullet -}$ caused by exposure to Topas. Ding et al. (2009) observed a dose-dependent significant decrease or increase in SOD activity in zebrafish (*Danio rerio*) after 96 h exposure to the triazole fungicide, paclobutrazol. By contrast, acute (96 h) exposure of *O. mykiss* to propiconazole decreased SOD activity in gills, whereas in liver, this parameter increased (Li et al., 2013). A decrease in SOD activity was reported in common carp fingerlings exposed to tebuconazole at concentrations of 1.78 and 2.37 mg L^{-1} (Toni et al., 2011).

The activity of the other primary antioxidant enzyme, catalase, was 12.7 ± 0.6, 181 ± 14 and 44.4 ± 2.0 U mg $protein^{-1}$ in gills, liver and kidney of control goldfish, respectively. In gills, catalase activity increased by 27% in fish exposed to 25 mg L^{-1} Topas, whereas in liver and kidney no statistically significant differences from control values were found (Fig. 3B). In this study, increased SOD and catalase activities in gills represent a commonly seen cascade of events in response to pesticide-induced oxidative stress that includes increases in the activities of antioxidant enzymes (Slaninova et al., 2009; Lushchak, 2011). Increased catalase activity was also observed in liver of *G. aculeatus* after treatment for 7 days with 500 μg L^{-1} prochloraz (Sanchez et al., 2008). In contrast to our results, the activity of catalase was significantly lower in gills of *O. mykiss* and higher in liver, as compared with corresponding controls, after acute (96 h) exposure to the triazole fungicide, propiconazole (Li et al., 2013). Significant increases in catalase activity were found in liver of rainbow trout after long term (20 and 30 d) exposure to propiconazole (Li et al., 2010a). A decrease in catalase activity was also described in fingerlings of *C. carpio* treated with 1.19 mg L^{-1} fungicide tebuconazole (Toni et al., 2011).

In our experiment, the activity of another H_2O_2-detoxifying enzyme, glutathione peroxidase (GPx), was found to be 72.4 ± 11.5, 223 ± 20, and 120 ± 6 mU mg $protein^{-1}$ in gills, liver, and kidney, respectively in control goldfish (Fig. 3C). Topas exposure did not affect GPx activity in gills and liver, at any of the concentrations used, whereas in kidney GPx activity increased by 12% in fish exposed to 15 or 25 mg L^{-1} fungicide as compared with controls (Fig. 3C). GPx is known to metabolize H_2O_2 and different organic hydroperoxides, such as LOOH, thereby minimizing oxidative damage to lipids (Lushchak, 2011). It was reported a decrease GPx activity in gills and increase in liver of *O. mykiss* after acute exposure to a fungicide propiconazole (Li et al., 2013). Li et al. (2010a) also showed a dose-dependent decrease of GPx activity in gills of rainbow trout after exposure to propiconazole for 30 days, whereas a significant increase in liver GPx activity was observed at the same experimental conditions.

3.3. Other glutathione-related enzymes

Several enzymes associated with glutathione recycling are involved in the antioxidant system response to any stressor. These include glutathione-S-transferase (GST) that conjugates glutathione with different

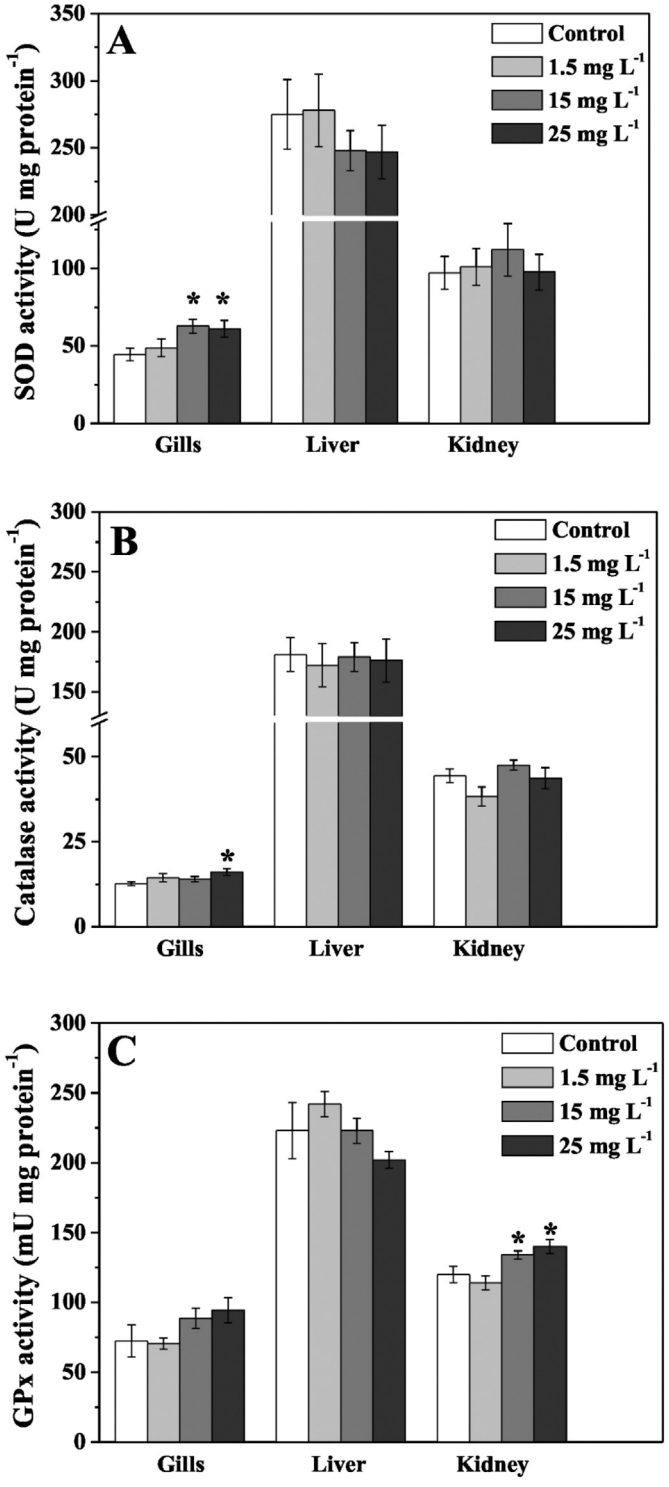

Fig. 3. The activities of superoxide dismutase (SOD, U mg protein^{-1}) (A), catalase (U mg protein^{-1}) (B), and glutathione peroxidase (GPx, mU mg protein^{-1}) (C) in gills, liver and kidney of goldfish, exposed to control conditions or 1.5, 15, and 25 mg L^{-1} of Topas for 96 h. Other information as in Fig. 1.

xenobiotics, glutathione reductase (GR) which maintains the glutathione pool in a highly reduced state (GSH), as well as glucose-6-phosphate dehydrogenase (G6PDH) that provides the pool of NADPH needed by the GR reaction (Lushchak, 2012).

Glutathione-*S*-transferase has a particularly important role in preventing free radical mediated oxidation of cellular components such as lipids and proteins (Seth et al., 2001). In the present study, total GST activity was highest in liver of the control goldfish (1.55 ± 0.09 U mg protein^{-1}), intermediate in kidney (1.15 ± 0.09 U mg protein^{-1}), and lowest in gills (0.60 ± 0.02 U mg protein^{-1}) (Fig. 4A). In gills, the activity of GST was elevated by 32 and 47% in fish exposed to 15 or 25 mg L^{-1} Topas, respectively. One can suggest that the detoxification pathway for penconazole in gills occurred via a direct conjugation of penconazole with GSH since its chemical structure favors such conjugation. This reaction increases the hydrophilicity of xenobiotics and

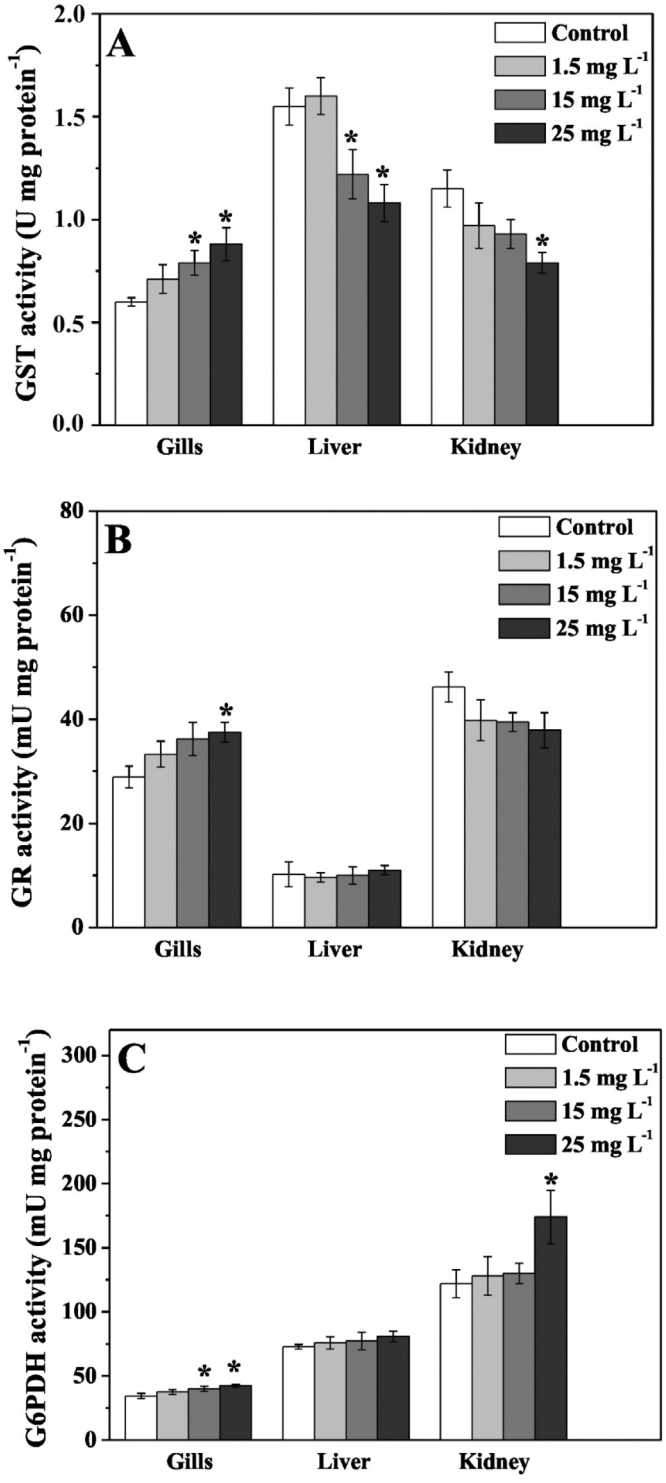

Fig. 4. The activity of glutathione-*S*-transferase (GST, mU mg protein^{-1}) (A), glutathione reductase (GR, mU mg protein^{-1}) (B), and glucose-6-phosphate dehydrogenase (G6PDH, mU mg protein^{-1}) (C) in gills, liver, and kidney of goldfish, exposed to control conditions or 1.5, 15, and 25 mg L^{-1} of Topas for 96 h. Other information as in Fig. 1.

thereby enhances their excretion (Sanchez et al., 2008). Similar results were observed in gills of goldfish exposed to the pesticide Tattoo (Kubrak et al., 2012). Goldfish exposure to 15 and 25 mg L^{-1} Topas resulted in significantly decreased GST activity in liver by 21 and 30%, respectively, whereas in kidney the activity was decreased by 31% after fish exposure to 25 mg L^{-1} fungicide (Fig. 4A). The decrease of GST activity could result from its inactivation by ROS. For example, it is well known that GST is sensitive to products of the Haber-Weiss reaction (Hermes-Lima and Storey, 1993). Previous studies also reported either increases or decreases in GST activity in tissues of fish exposed to different triazole fungicides (Egaas et al., 1999; Zhu et al., 2014; Li et al., 2010a; Tu et al., 2016). For example, an increase in GST activity was also observed in liver of three-spined sticklebacks after 7 and 14 days exposure to prochloraz (Sanchez et al., 2008). However, a significant decrease in GST activity was observed in developing rare minnow embryos exposed to 5.0 mg L^{-1} of different triazole fungicides (myclobutanil, fluconazole, flusilazole, triflumizole, and epoxiconazole) (Zhu et al., 2014). Common carp fingerlings exposed to tebuconazole showed a significant decrease in GST activity when compared to the control group (Toni et al., 2011). GST activity was significantly increased in liver of rainbow trout after exposure to propiconazole for 20 and 30 days, but activity in gills was reduced (Li et al., 2010a). Up-regulation of GST activity may be one of the adaptive mechanisms of mild oxidative stress induced by penconazole (Lushchak, 2014). Additionally, GST induction has been suggested as a biomarker of specific cancers (Tew, 1994) and the related antioxidant system in fish could be useful for monitoring of residual fungicides in the aquatic environment.

Glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH) are auxiliary enzymes in antioxidant defense: GR reduces oxidized glutathione (GSSG) to its active form, reduced glutathione (GSH), whereas G6PDH provides the reducing power (NADPH) needed for the GR reaction (Lushchak, 2012, 2014). The activities of GR in control fish were 28.9 ± 2.1, 10.2 ± 2.4, and 46.2 ± 2.9 mU mg protein^{-1} in gills, liver, and kidney, respectively. Fish exposure to Topas at the highest concentration used (25 mg L^{-1}) resulted in enhanced GR activity in gills by 30%, as compared with controls (Fig. 4B). However, GR activity was unchanged in the other two tissues, suggesting that their constitutive activities in liver and kidney are probably sufficient to deal with any oxidative stress arising from Topas exposure (Fig. 4B). Increased GR activity in gills in response to the highest Topas concentration correlated with an enhanced concentration of L-SH (Fig. 2B), indicating that this enzyme was probably responsible for raising the reduced glutathione pool, that contribute to higher level of L-SH. Long-term (20–30 days) exposure to propiconazole also induced a significant increase in the activity of GR in liver of *O. mykiss*, but its significant decrease was observed in gills under the same experimental conditions (Li et al., 2010a). A significantly higher activity of GR was also observed in liver of rainbow trout, as compared with control values, after acute exposure to propiconazole, whereas in gills the activity of this enzyme was lower than those in the control group (Li et al., 2013).

Glucose-6-phosphate dehydrogenase (G6PDH) activity also demonstrated tissue specificity. Maximal activity among the tissues studied was observed in kidney of control goldfish (122 ± 11 mU mg protein^{-1}), and was lower in liver and gills, 72.7 ± 1.80 and 34.3 ± 2.00 mU mg protein^{-1}, respectively (Fig. 4C). In gills, G6PDH activity was enhanced by 16 and 23% in fish exposed to Topas at 15 and 25 mg L^{-1}, respectively. The most substantial increase in G6PDH activity was observed in kidney of goldfish after exposure to 25 mg L^{-1} Topas, where the activity exceeded the control value by 43%. In contrast, the G6PDH activity was virtually the same in liver of fish exposed to any Topas concentration (Fig. 4C). The elevated G6PDH activity could support higher NADPH production in gills and kidney to meet the needs of some antioxidant defenses or biosynthetic processes in these tissues under Topas exposure. Previously, we also found increased activity of this enzyme in kidney of goldfish exposed to the metribuzin-containing pesticide Sencor (Husak et al., 2014).

3.4. Potential mechanisms responsible for penconazole-induced stress

Xenobiotics include drugs, food additives, environmental pollutants, and fungal or microbial toxins making up hundreds of thousands of compounds affecting cellular integrity. Exposure to these foreign compounds is known as xenobiotic induced stress. To cope with this stress, all groups of organisms possess particular metabolic pathways that detoxify potentially hazardous compounds. However, intermediates and end products of xenobiotic metabolism may become toxic as well, and frequently give rise to ROS formation (Pagano, 2002).

Penconazole is known to inhibit sterol biosynthesis in fungi. However, sterol biosynthesis is an essential metabolic pathway in animals (cholesterol), fungi (ergosterol) and plants (sitosterol) and requires the removal of the C32-methyl group at position 14α- from precursor sterols. This reaction is catalyzed by a microsomal cytochrome P_{450}, the lanosterol 14α-demethylase (CYP51) (Korošec et al., 2008). The dichlorinated phenyl group of penconazole can form a van der Waals bond with the hydrophobic residues of amino acids of the Cyp51 active site (hydrophobic pocket). The nitrogen atom of the five-membered aromatic ring of penconazole may bind to the heme moiety and to the serine residue at the active site of Cyp51 (Fig. 5). In addition, penconazole can form water-bridging interactions at the active site (Snelders et al., 2012). Blocking the fish CYP51 activity with penconazole may result in inhibition of cholesterol biosynthesis.

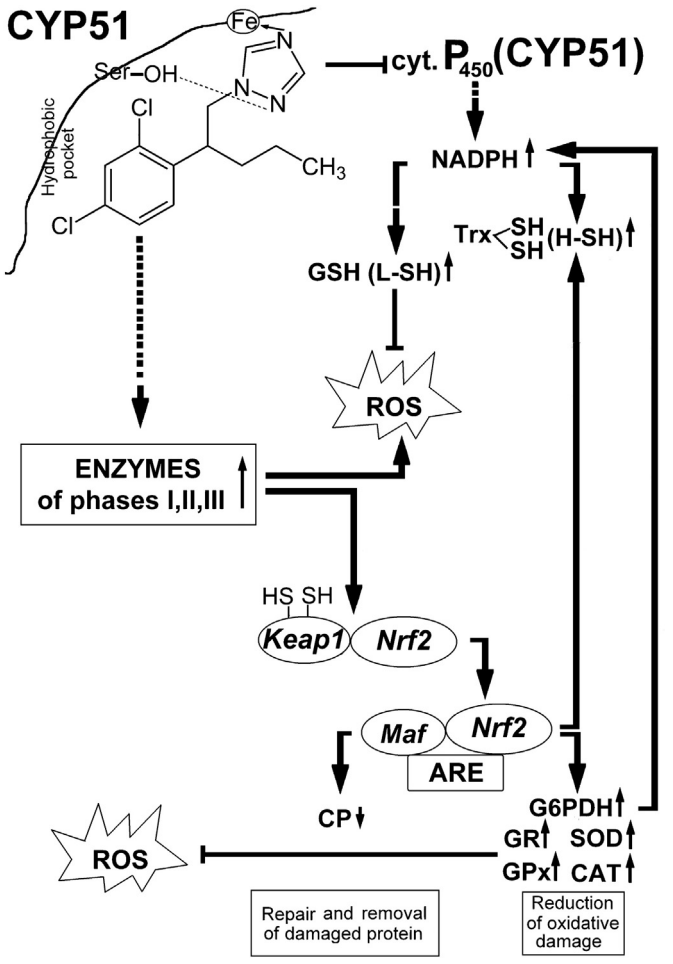

Fig. 5. Hypothetical representation of mechanisms responsible for penconazole-induced stress in fish. CYP51, P450-dependent 14α-demethylase; ROS, reactive oxygen species; CP, carbonyl protein; L-SH, low molecular mass thiols; H-SH, high molecular mass thiols; SOD, superoxide dismutase; CAT, catalase; GR, glutathione reductase; G6PDH, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; GSH, glutathione; Nrf2, erythroid-derived-2-like 2 transcription factor; Keap1, ECH-associated protein 1; ARE, antioxidant response element; Trx, thioredoxin.

Detoxification of penconazole compounds is supposed to include three phases (Wolterink and Inayat-Hussain, 2015). *Phase I* of penconazole biotransformation includes cleavage of the triazole ring, oxidation of the ω-position of the alkane chain to form the respective carboxylic acid, oxidation of the 3- or 4-position of the alkane chain to form monohydroxy and dihydroxy derivatives, and oxidation of the triazole ring at the 3- or 5-position. Secondary metabolic reactions (*Phase II*) include α-oxidation of the carboxylic acids to form α-hydroxy carboxylic acids, decarboxylation following oxidation to an α-ketocarboxylic derivative, oxidation of the 3,4-dihydroxy derivatives to produce the corresponding 3- or 4-keto derivatives, and conjugation of all alkanol derivatives with glucuronic acid. In *Phase III*, these products are exported from the cells by ABC/MRP or ABC/MDR transporters (Wolterink and Inayat-Hussain, 2015).

Notably, many phase I, II, and III enzymes are target genes of the erythroid-derived-2-like 2 transcription factor (Nrf2). Increased Nrf2 activity has often correlated with cellular survival and protection. Normally, Nrf2 is held in an inactive state in the cytoplasm by binding to Kelch-like ECH-associated protein 1 (Keap1); binding can lead to Nrf2 degradation via the proteasome (Howden, 2013; Suzuki et al., 2013). Under conditions of oxidative/electrophilic or xenobiotic stress, the Cullin3/Rbx1-dependent polyubiquitination of Nrf2 under the assistance of Keap1 is blocked. Nrf2 is then free to move from the cytoplasm into the nucleus where it binds to the small Maf protein and the antioxidant response element (ARE) of DNA. Activation of the ARE leads to the transcriptional activation of multiple antioxidant enzymes and proteins, including SOD, catalase, thioredoxin 1 (Trx1), GR, GPx, and G6PDH (Kaspar et al., 2009). As summarized in Fig. 5, our results allow us to suggest that the penconazole-containing fungicide Topas activates the Nrf2 pathway and induces expression of antioxidant and related enzymes, thereby providing enhanced protection to cells from oxidative stress-induced apoptosis, preventing ROS over-production, and inhibiting protein oxidation. In this study, we observed significant increases of enzyme activities including SOD, catalase, GR, GPx, G6PDH in different tissues of goldfish after Topas exposure (Figs. 3 and 4). All of these enzymes are distinguished by their ability to prevent or minimize ROS-promoted oxidative damage. In our experiment, the activity of G6PDH was increased in gills and kidney after Topas exposure (Fig. 4C) which could enhance NADPH levels and lead to higher GSH content (Fig. 5). The described cascade response to the pesticide might be initiated by enhanced ROS production associated with either penconazole biotransformation or autoxidation of its hydroxylated metabolites. Collectively, the results of this research allow us to conclude that at low concentrations Topas may augment antioxidant defense mechanisms via induction of mild oxidative stress leading to up-regulation of antioxidant mechanisms, whereas at high concentrations it may induce severe oxidative stress which may be responsible for the toxicity of Topas to fish.

4. Conclusions

Our results clearly demonstrate that goldfish exposure to Topas, at least at the concentrations used, does not cause substantial oxidative stress, but led to perturbations of ROS homeostasis in goldfish gills, liver, and kidney in different manners. Perturbations of ROS-related processes in response to goldfish exposure to Topas were most pronounced in gills among the three tissues analyzed. The high basal level of CP was decreased under Topas-induced stress may be due to elevated activities of SOD, catalase, GST, GR and G6PDH, as well as increased level of thiol-containing compounds. Of the three tissues tested, goldfish liver possesses the most powerful antioxidant defense system as evidenced by increased levels of L-SH and H-SH under stress condition and high constitutive activities of SOD, catalase, GPx, and GST. In kidney, only four of 10 parameters measured were affected at the highest Topas concentration used: L-SH levels and activities of GPx, GST and G6PDH, indicative of a relatively weak antioxidant defense response in this organ. It is supposed that animal exposure to Topas at the selected concentrations induced weak oxidative stress that resulted in up-regulation of antioxidant defense (Lushchak, 2014). Thereby, this prevented an increase in lipid peroxide levels and even decreased protein carbonyls, both being broadly accepted markers of oxidative stress. Future studies should explore the evidence for a hypothesis of the development of reductive stress in goldfish gills upon treatment with penconazole-containing fungicides. The obtained results call for this discussion and open new avenues for future investigations.

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