Pro-oxidant potency of clothianidin in rainbow trout

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Clothianidin is a systemic neonicotinoid insecticide interfering with the central nervous system by acting as a nicotinic acetylcholine receptor agonist. Although previous studies on fish report low toxicity, its proven toxic potential for aquatic invertebrates and lack of data on its effect on juvenile fish have prompted us to investigate its adverse effects in environmentally relevant concentrations of 3, 15 and 30 µg/L for 7, 14 and 21 days on heart and spleen tissues of 10-month-old rainbow trout (Oncorhynchus mykiss). We detected a conspicuous increase in protein carbonyl and malondialdehyde (MDA) levels, which triggered antioxidant response of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), resulting in increased levels of glutathione (GSH). Clothianidin inhibited the activity of acetylcholinesterase (AChE) and lowered tissue protein levels. Heart tissue weight increased, while that of spleen decreased significantly. The effects were time- and concentration-dependent. What raises particular concern is the inhibition of AChE in the trout, even though clothianidin is claimed to be selective for insect receptors. Increased antioxidant activity in response to oxidative stress was clearly insufficient to keep MDA and protein carbonyl at normal levels, which evidences the pro-oxidant potency of the insecticide. All this calls for further investigation into potential adverse effects on biological pathways in different fish species.

KEY WORDS: AChE; CAT; fish; GPx; GSH; heart; MDA; neonicotinoids; Oncorhynchus mykiss; oxidative stress; SOD; spleen

Neonicotinoids are an important class of systemic insecticides, whose use for crop protection against piercing and sucking insects and animal health care started in the early 1990s. Their high solubility in water and slow breakdown in soil makes them readily absorbed by plants and has raised concern about leaching and transport in bodies of water (1), which is why they are listed as common contaminants of surface waters at concentrations in the µg/L range (2).

Neonicotinoids act as agonists of nicotinic acetylcholine receptors (nAChRs), which play a crucial role in the synaptic transmission in the central nervous system of insects (3, 4). Due to their selectivity for insect receptors, they are believed to exert low toxicity in vertebrates, but growing recent evidence points to their ability to affect the vital functions of non-target species such as fish and mammals (5–7).

Clothianidin [(E)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl2-nitroguanidine; CAS No. 210880-92-5] is the second commercialised neonicotinoid (the first was imidacloprid) and is one of the most polar and reactive members of the group. Its persistence and mobility in the ground and surface waters (8, 9) have resulted in its ban of use in flowering crops that appeal to honey bees (along with thiamethoxam and imidacloprid) by the European Commission (10) in 2013 and intensified investigations (11–13). In 2019, the United States Environmental Protection Agency (US EPA) cancelled its registration along with 11 other neonicotinoids, permitting the sale and distribution of leftover stocks until 20 May 2020 and regulating safe disposal beyond this deadline (14).

In the meantime, research of adverse effects of clothianidin moved to non-target aquatic invertebrate species (15, 16) and to the fish as one step up the food web, which resulted in a revision of its previously non-toxic status to slightly toxic (17). Investigation broadened to include various aspects of clothianidin toxicity, including neurotoxicity (18, 19) and behavioural impairment in rats (20) and oxidative stress and reproductive toxicity in rats, birds, and fish (21–23). However, as little is still known about its adverse effects in young fish, we decided to fill this gap by investigating the vulnerability of young rainbow trout (Oncorhynchus mykiss) to clothianidin toxicity by evaluating its effects through (i) anticholinesterase (AChE) activity, and (ii) oxidative stress in their heart and spleen tissues.

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MATERIALS AND METHODS

Study species and water conditions

The choice of trout was based on earlier evidence that this fish is highly sensitive to environmental changes (24) and can serve as a reliable bioindicator of acute and chronic toxicity (25). We used 48 10-month-old specimens (weighing 139.02±15.26 g and 19.23±1.68 cm long), purchased from a local breeder (Tunc Alabalık-Sanliurfa, Birecik, Turkey) and acclimatised to laboratory conditions for two weeks. They were held in 150 L water tanks with aerated tap water (under the 12/12 h dark/light cycle) and fed daily with commercial trout food (Optima trout-Skretting, Mugla, Turkey) at a rate of 2 % of their body weight throughout acclimatisation and exposure to clothianidin, except for the last 24 h before killing and immediate dissection.

The physico-chemical properties of water were as follows: temperature 16.96±0.32 °C, pH 7.5±0.34, dissolved oxygen 8.5±0.67 mg/L, and conductivity 731.25±14.34 µS/cm. These were maintained and checked twice daily with a multi-parameter water meter (84051 AZ Instrument Corp., Taichung City, Taiwan) before and after renewal of test solution (42 measurements in total).

The experimental protocols were authorized by the local Ethics Committee of Animal Experiments of Gaziantep University, under protocol No. 92 and decision No. 2019/13.

Experimental design

Following acclimatisation, the trout were divided into four groups of 12. One served as control and other groups were exposed to three sublethal and environmentally relevant concentrations of analytical standard (≥98 % purity) clothianidin (PESTANAL™, MilliporeSigma Supelco, Munich, Germany) falling within reported ranges in water sources (2, 26–28), namely 3, 15, and 30 µg/L. To obtain desired concentrations in the water tanks, clothianidin, except for the last 24 h before killing and immediate dissection.

Subacute toxicity was determined at 7-day sampling intervals (on days 7, 14, and 21).

Sampling and analysis

We determined oxidative stress through its adverse effects on lipids (lipid peroxidation) and proteins (protein carbonylation), activity of antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and the level of glutathione (GSH) as radical scavenger (30–33), while the selection of the heart and spleen tissues in which they were measured was based on their role in general fitness and adaptation to environmental conditions (heart) and the function of the immune system (spleen).

At the end of each exposure period, four randomly selected fish from each tank were anaesthetised with tricaine mesylate (MS-222, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and euthanised by spinal cord section. Heart and spleen were carefully removed, washed with ice-cold physiological saline (0.59 % NaCl), weighted, and stored at -80 °C until analysis. The weights of each heart and spleen were used to calculate heart and spleen somatic indexes (HSI and SSI) according to following formula:

\[ \text{Organsomatic index} = \frac{\text{tissue weight}}{\text{body weight} \times 100} \]

Frozen tissue samples were homogenised with 0.1 mol/L pH 7.4 phosphate buffer saline (PBS) (1:5 w/v, 0.2 g tissue/1 mL buffer) and centrifuged at 9744 g and 4 °C for 30 min (Hettich Universal 320R, Sigma-Aldrich) to obtain post-mitochondrial supernatants.

MDA, GSH, and protein carbonyl were analysed with respective commercial enzyme-linked immunosorbent assay (ELISA) kits (Fish Malondialdehyde ELISA Kit, Cat. No E0017Fi; Fish Glutathione ELISA Kit, Cat. No EA0037Fi; Fish Protein Carbonyl ELISA Kit, Cat. No E0112Fi; Bioassay Technology Laboratory, Shanghai, China) using the quantitative sandwich method according to the manufacturer’s instructions. Optical density was measured at 450 nm with a Thermo Scientific Multiscan GO microplate reader (Thermo Fisher Scientific Oy, Ratakaste, Finland) and the quantity calculated from the standard curve. The standard curve range for MDA was 0.05–30 nmol/mL, for protein carbonyl 1–400 ng/mL, and the sensitivity for GSH was 0.25 nmol/mL.

SOD, CAT, GPx, and AChE activities and protein levels in the supernatants were determined spectrophotometrically (Shimadzu UV Mini-1240, Shimadzu Corporation, Duisburg, Germany). SOD activity was analysed as described by McCord and Fridovich (35) through iodonitrotetrazolium chloride (INT, Sigma-Aldrich) reduction monitored at 505 nm. The assay mixture was as follows: 0.01 mol/L phosphate buffer (pH 7.0), 80 U/L xanthine oxidase (Sigma-Aldrich), 0.05 mmol/L xanthine (Sigma-Aldrich), 0.025 mmol/L INT, and 1 mL of tissue homogenate.

GPx activity was measured by monitoring dismutation of t-butylhydroperoxide (Sigma-Aldrich) at 340 nm (36). The assay mixture contained 0.5 mol/L Tris buffer (pH 8.0), 0.1 mol/L GSH, 10 U/mL of glutathione reductase (Sigma-Alaldrich), 2 mmol/L NADPH (Sigma-Aldrich), 7 mmol/L t-butylhydroperoxide, 660 µL of distilled water, and 1 mL of tissue homogenate.

CAT activity was measured through the degradation rate of hydrogen peroxide at 230 nm in a reaction mixture of 1 mol/L Tris buffer (pH 8.0), 10 mmol/L H₂O₂, 30 µL distilled water, and 20 µL tissue homogenate (36).
AChE activity was measured according to Ellman et al. (37) by recording absorbance at 412 nm in a reaction mixture of 0.1 mol/L pH phosphate buffer (pH 8.0), 0.015 mol/L acetylthiocholine iodide (Sigma Aldrich), 8.52 mmol/L ethopropazine (Sigma Aldrich), and 0.01 mol/L 5,5-dithiobis-2-nitrobenzoic acid (DTNB, Sigma-Aldrich).

Tissue protein levels were determined according to the method described by Lowry et al. (38) using bovine serum albumin (Sigma Aldrich) as standard.

Statistical analysis

All data were analysed using the SPSS v. 22 software (SPSS Inc., Chicago, IL, USA). Their distribution was checked for normality (Q-Q plot) and homogeneity of variance (Levene’s test), respectively. Intergroup differences were assessed using one-way analysis of variance (ANOVA) followed by post-hoc least-significant difference test (LSD) (p<0.05). The strength of association between variables was tested with Pearson’s correlation analysis. Results are given as means ± standard error.

RESULTS

Changes in the analysed parameters following clothianidin exposure are presented as percentages of control.

Organosomatic indices

Heart and spleen somatic indices did not significantly differ from control on days 7 and 14 of exposure (Table 1). Significant changes were observed at 30 µg/L on day 21. Heart somatic index increased 45 % and spleen somatic index decreased 55 % (p<0.05).

Protein levels

In heart tissue, clothianidin exposure resulted in a 48 % reduction (p<0.01) in tissue protein level on day 21, while no significant changes were observed on days 7 and 14. A similar decreasing trend was observed for spleen tissue, with the highest reduction rate of 42 % and 47 % on days 14 and 21 (p<0.05) (Table 2).

GSH

GSH levels did not change significantly on days 7 and 14, but increased 216 % on day 21 at 30 µg/L in heart tissue (p<0.01). In spleen tissue, the only significant change was the 90 % increase at the 30 µg/L concentration on day 14 (p<0.01) (Table 2).

Protein carbonyl

Protein carbonyl levels showed an increase of 70 %, 69 %, 184 % in heart tissue on respective days 7, 14, and 21 of exposure to clothianidin at 30 µg/L (p<0.05). In spleen tissue, the same concentration increased protein carbonyl levels 32 % and 56 % on days 14 and 21 (p<0.01) (Table 2).

MDA

In heart tissue, MDA levels increased 150 %, 81 %, and 371 % at the highest pesticide concentration on days 7, 14, and 21 (p<0.05). In spleen tissue, MDA levels significantly increased on days 14 and 21, 54 % and 63 %, respectively (p<0.01) (Table 2).

Antioxidant enzyme activities

Figure 1 shows changes in antioxidant enzyme activities in heart and spleen tissues. SOD sharply increased (198 %) only on day 21 in heart tissue, regardless of clothianidin concentration (p<0.01). In spleen, it increased earlier at all tested concentrations, and the highest increase was 123 % (p<0.01).

Similarly, heart CAT activity increased on day 21, reaching the maximum of 165 % (r=0.795, p<0.01). Spleen

Table 1 Heart and spleen somatic indices in rainbow trout by weeks of exposure to clothianidin

| Somatic indices | 7 days | 14 days | 21 days |
|-----------------|--------|---------|---------|
| **Heart**       |        |         |         |
| Control         | 0.119±0.015a | 0.126±0.008ax | 0.123±0.008xy |
| 3 µg/L          | 0.139±0.005ax | 0.111±0.012ax | 0.137±0.014ax |
| 15 µg/L         | 0.155±0.030a | 0.149±0.021ax | 0.158±0.024ax |
| 30 µg/L         | 0.125±0.004ax | **0.147±0.019**ay | **0.178±0.013**by |
| **Spleen**      |        |         |         |
| Control         | 0.204±0.053ax | 0.225±0.048ax | 0.206±0.010ax |
| 3 µg/L          | 0.172±0.037ax | 0.147±0.035ax | 0.106±0.022ax |
| 15 µg/L         | 0.287±0.065ax | 0.154±0.030ax | 0.113±0.012ax |
| 30 µg/L         | 0.197±0.036ax | 0.214±0.055ax | **0.092±0.009**by |

Values are given as mean ± standard error (n=4). Superscript letters a and b indicate differences between exposure concentrations; superscript letters x and y indicate differences between durations (p<0.05)
tissue activity statistically rose at 30 µg/L on day 14 and at all tested concentrations on day 21 (max. 168 %, p<0.05).

GPx heart tissue activity also waited to significantly rise until day 21 at the highest clothianidin concentration (max. 194 %, p<0.01). In spleen tissue, antioxidant response to the highest concentration came earlier to reach 118 % (max. 194 %, p<0.01). In spleen tissue, antioxidant response statistically rose at 30 µg/L on days 14 and 21 in heart tissue (25 % and 44 %, respectively, p<0.01) and on day 21 in spleen (27 %, p<0.05).

**AChE activity**

AChE activities started to significantly drop only at 30 µg/L on days 14 and 21 in heart tissue (25 % and 44 %, respectively, p<0.01) and on day 21 in spleen (27 %, p<0.05).

**Correlations**

Table 3 shows Pearson’s correlation coefficients for all heart and spleen tissue parameters. Protein carbonyl and GSH levels and all antioxidant activities positively correlated with MDA in both tissues (p<0.01). AChE activity negatively correlated with MDA levels (p<0.01) only in heart tissue.

**DISCUSSION**

Our findings clearly evidence that environmentally relevant concentrations of clothianidin can induce oxidative stress, deplete protein levels, and inhibit AChE activity in time- and concentration-dependent manner.

The same is true for its effects on heart and spleen sizes. Heart somatic index increased, and spleen somatic index decreased. The observed heart enlargement reflects its susceptibility to cardiotoxic chemicals (39, 40) and may be explained by cardiac hypertrophy as an adaptive response to prolonged stress (41). Similar effects on spleen were explained by cardiac hypertrophy as an adaptive response to increased MDA levels (p<0.01), possibly indicating increased proteolysis and/or energy requirements in response to a stressful condition, as reported elsewhere (46–48).
Our AChE findings seem to support other studies which warn about the fallacy that clothianidin (and generally neonicotinoid) anticholinesterase activity is minimal in non-target organisms (49, 50). While some studies found no relevant changes in AChE activity, such as the one on imidacloprid effect in brain and muscle tissues of exposed streaked prochilod (Prochilodus lineatus) (51), others report significant changes with prolonged exposure (52, 53).

Earlier response to oxidative stress by spleen than heart tissue evidenced by increase in SOD activity points to its greater vulnerability. Concomitant rise in CAT and GPx activities and the observed changes in GSH levels evidence a disturbance in cellular $H_2O_2$. This increase was likely caused by increased transcription and/or translation due to lack of the posttranslational regulation (54) and reveals the pro-oxidant activity of clothianidin in juvenile rainbow trout. Similar findings were reported in other fish species after prolonged exposure to neonicotinoids (55–58).

Even though the activities of antioxidant enzymes and GSH levels rose, they could not mitigate oxidative stress, as MDA in both tissues remained high. Similar findings were also reported for other fish exposed to neonicotinoids (52, 53).

Increased protein carbonyl levels reinforced our findings of increased oxidative stress in both tissues caused by clothianidin. Protein carbonyls are reliable biomarkers of oxidative stress and usually follow changes in MDA (59, 60), and our findings support earlier reports on pesticide effects in fish (61–63).

CONCLUSION

Besides clearly showing that environmental concentrations of clothianidin can adversely affect young rainbow trout in a concentration- and time-dependent manner, this research provides an insight into its toxic mechanisms of action. The limitation inherent to the study design, however, is that it does not reflect real-life co-exposure to other chemicals present in aquatic ecosystems, which may have additive or synergistic effects. Therefore, future studies should focus on an integrated risk

**Figure 1** Change in mean SOD, CAT, and GPx enzyme activities in heart and spleen tissues of rainbow trout following clothianidin exposure with respect to control. * p<0.05; ** p<0.01

**Figure 2** Change in mean AChE enzyme activity in heart and spleen tissues of rainbow trout following clothianidin exposure with respect to control. * p<0.05; ** p<0.01
Table 3 Pearson correlation coefficients for heart and spleen tissue biomarkers in rainbow trout following clothianidin exposure

| Protein          | Protein carbonyl | MDA   | AChE   | SOD    | CAT    | GPx    | GSH    |
|------------------|------------------|-------|--------|--------|--------|--------|--------|
| Heart            |                  |       |        |        |        |        |        |
| Protein          | 1                |       |        |        |        |        |        |
| Protein Carbonyl | -0.788*          | 1     |        |        |        |        |        |
| MDA              | -0.760*          | 0.901*| 1      |        |        |        |        |
| AChE             | 0.516*           | -0.633*| -0.654*| 1      |        |        |        |
| SOD              | -0.816*          | 0.874*| 0.930*| -0.582*| 1      |        |        |
| CAT              | -0.671*          | 0.781*| 0.809*| -0.437*| 0.735*| 1      |        |
| GPx              | -0.874*          | 0.849*| 0.937*| -0.598*| 0.951*| 0.798*| 1      |
| GSH              | -0.855*          | 0.908*| 0.942*| -0.624*| 0.965*| 0.792*| 0.969*| 1      |
| Spleen           |                  |       |        |        |        |        |        |
| Protein          | 1                |       |        |        |        |        |        |
| Protein Carbonyl | -0.526*          | 1     |        |        |        |        |        |
| MDA              | -0.705*          | 0.650*| 1      |        |        |        |        |
| AChE             | -0.254           | 0.225 | 0.218 | 1      |        |        |        |
| SOD              | -0.765*          | 0.505*| 0.758*| 0.265  | 1      |        |        |
| CAT              | -0.736*          | 0.647*| 0.749*| 0.141  | 0.725*| 1      |        |
| GPx              | -0.789*          | 0.589*| 0.818*| 0.239  | 0.924*| 0.723*| 1      |
| GSH              | -0.664*          | 0.576*| 0.832*| 0.165  | 0.853*| 0.597*| 0.927*| 1      |

*p<0.01 (Pearson correlation analysis, 2-tailed)

assessment of combined environmental exposure through combined treatment or a mesocosm experiment. Additional studies with fish at different life stages, including larval and embryonic, could shed more light of the mechanisms of action.

Conflicts of interest

None to declare.

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Prooksidacijski potencijal klotianidina u kalifornijske pastrve

Klotianidin je sistemski neonikotidni insekticid koji utječe na središnji živčani sustav kao agonist nikotinskoga receptora za acetilkolin. Iako su ranija istraživanja pokazala njegovu nisku toksičnost u riba, njegova dokazana toksičnost u vodenih beskralježnjaka i nedostatak istraživanja o njegovu djelovanju u mladim riba potaknuli su nas da istražimo njegove štetne učinke na tkivo srca i slezene 10-mjesečnih kalifornijskih pastrva (*Oncorhynchus mykiss*), izloženih okolišnim koncentracijama od 3, 15 i 30 µg/L u trajanju od 7, 14 i 21 dan. Vidljiv je bio porast razina proteinskih karbonila i malondialdehida (MDA), koji je potaknuo pojačanu antioksidacijsku aktivnost superoksid dismutaze (SOD), katalaze (CAT) i glutation peroksidae (GPx) te doveo do povećanja razina glutationa (GSH) u tkivima. Pritom je inhibirao aktivnost acetilkolinesteraze (AChE) i smanjio razine proteina. Masa srčanog tkiva se povećala, a ona slezene značajno se smanjila. Učinci su bili ovisni o koncentraciji i trajanju izloženosti. Posebice zabrinjava inhibicija AChE u pastrva, premda se smatra da klotianidin cilja isključivo nikotinske receptore za acetilkolin u insekata. Povećana antioksidacijska aktivnost kao odgovor na oksidacijski stres bila je nedostatna da MDA i proteinske karbonile zadrži na normalnim razinama, što upućuje na snažno prooksidacijsko djelovanje ovog insekticida. Zbog toga je potrebno podrobnije istražiti njegove moguće štetne učinke na biološke puteve u različitim ribljim vrstama.

KLJUČNE RIJEČI: AChE; CAT; GPx; GSH; MDA; neonikotinoidi; *Oncorhynchus mykiss*; oksidacijski stres; riba; slezena; SOD; srce