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Oxidative and apoptotic effects of fluoxetine and its metabolite norfluoxetine in *Daphnia magna*

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The aim of this study was to investigate the oxidative and apoptotic potential of fluoxetine, a widely used antidepressant in Turkey and the world, and of its metabolite norfluoxetine on a model non-target organism, *Daphnia magna* to see how exposure to this group of antidepressants (specific serotonin reuptake inhibitors) could affect the aquatic environment in which they end up. Juvenile *D. magna* specimens were chronically exposed to fluoxetine and norfluoxetine alone and in combination at concentrations found in the aquatic environment (0.091 and 0.011 µg/L, respectively) and to their 10-fold environmental concentrations for 21 days. Another group of 17-day-old animals were subacutely exposed to 100-fold environmental concentrations for four days. After exposure, we measured their glutathione peroxidase (GPx) and cholinesterase (ChE) activities, thiobarbituric acid-reactive substances (TBARS), and total protein content spectrophotometrically, while mitochondrial membrane potential (MMP) was analysed by fluorescence staining, and cytochrome c and ERK1/2 protein content by Western blotting. This is the first-time cytochrome c and ERK1/2 were determined at the protein level in *D. magna*. We also measured their carapace length, width, and caudal spine length microscopically. At environmental concentrations fluoxetine and norfluoxetine caused an increase in ChE activity and brood production. They also caused a decrease in juvenile carapace length, width, and caudal spine length and depolarised the mitochondrial membrane. At 10-fold environmental concentrations, GPx activity, lipid peroxidation levels, cytochrome c, and ERK1/2 protein levels rose. The most pronounced effect was observed in *D. magna* exposed to norfluoxetine. Norfluoxetine also decreased brood production. Similar effects were observed with subacute exposure to 100-fold environmental concentrations. However, total protein content decreased. All this confirms that fluoxetine and norfluoxetine have oxidative and apoptotic potential in *D. magna*. *Daphnia spp.* have a great potential to give us precious insight into the mechanisms of environmental toxicants, but there is still a long way to go before they are clarified in these organisms.

KEY WORDS: cholinesterase; cytochrome c; ERK1/2; GPx; lipid peroxidation; mitochondrial membrane potential; oxidative stress; TBARS

Recent times have seen a growing concern about the effects of waterborne pharmaceuticals on human and ecosystem health as their detection in water has advanced to as low as nano-levels (1). Their effects on non-target organisms, however, have hardly been investigated so far (2, 3). Usually they contaminate drainage waters as the main compound, its metabolites, and/or conjugates at micromolar levels (4). Being relatively polar, they cannot be removed from waters easily. Psychotherapeutics such as antidepressants, cardiovascular drugs, and anti-infectives seem to stand out as particularly dangerous to aquatic life (5, 6).

In this study we focused on one particular antidepressant acting through inhibition of serotonin reuptake, that is, fluoxetine (Flx; IUPAC name: *N*-methyl-3-phenyl-3-(4-(trifluoromethyl) phenoxy) propan-1-amine; CAS No: 56296-78-7) (7), as Turkey has seen an alarming increase in anti-depressant use between 2008 and 2018 (from 27.1 to 44.1 daily doses per 1000 persons) (8). Its main metabolite norfluoxetine (NorFlx; IUPAC name: 3-phenyl-3-(4-(trifluoromethyl) phenoxy) propan-1-amine; CAS No: 83891-03-6) (9) also caught our attention as potentially harmful to the aquatic environment, as it restores its non-glucuronide form in water treatment plants (10). The two compounds have been reported in concentrations as high as 0.091 µg/L for Flx and 0.011 µg/L for NorFlx in a wastewater treatment plant in Grand River, Ontario, Canada (11), and there are other reports of their detection in water all over the world (12, 13).

Fluoxetine can bioaccumulate in *Daphnia magna* (14) and affect different physiological pathways in this species because of the presence of serotonin and functionally related proteins in this genus (15, 16). It can affect the morphology and brood production rate in *D. magna* and also have

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oxidative effect in this species (14). Its toxicity has generally been evaluated at higher concentrations than those found in the environment, and there is no information about the effects of its metabolite NorFlx.

In this study we considered three scenarios to evaluate the toxicity of Flx, NorFlx, and their combinations: chronic exposure to a realistic environmental concentration; chronic exposure to a 10-fold environmental concentration, and subacute exposure to a 100-fold environmental concentration because of increased antidepressant use in the world. Besides morphological and brood production changes, we evaluated oxidative and anticholinesterase effects of Flx, NorFlx and their combinations in these three scenarios on cytochrome c and ERK1/2 protein levels and mitochondrial membrane polarisation (MMP).

MATERIALS AND METHODS

D. magna specimens were purchased from Carolina Biological Supply Company (Burlington, NC, USA) and were acclimatised to laboratory conditions in 15 L of hard water (American Society for Testing and Materials, ASTM) in plastic containers for a minimum of ten generations (17). The physicochemical properties of hard water were as follows: pH 8.00, 20.20±0.35 °C, total alkalinity 120.42±7.77 mg/L $CaCO_3$, total hardness 138.51±8.52 mg/L $CaCO_3$, and light intensity 10.53 µmol/m^2/s. The light-to-dark photoperiod was 16:8. Animals were fed every other day with a 1.5 mL/L mixture of trout chow, yeast, and alfalfa (18). Before the toxicity experiments, the fifth-generation mothers from one mother were used as the source of experimental organisms. Less than 24 h-old second-generation offspring of these mothers were used in the 24 h $K_2Cr_2O_7$ EC_{50} susceptibility test. The half maximal effective concentration (EC_{50}) was 0.972 mg/L (95 % confidence interval was 0.904–1.042) and was in the range proposed by the Organisation for Economic Co-operation and Development (OECD) (19).

Fluoxetine hydrochloride (Supelco PHR1394) and its human metabolite norfluoxetine hydrochloride (Supelco 40724) were obtained from Supelco, Inc. (Bellefonte, PA, USA). Serial dilution method was used to prepare the exposure media. The efficiency of our dilution method was confirmed by liquid chromatography with tandem mass spectrometry (LC-MS/MS). 100 µL of water sample was injected into the LC-MS/MS device (LCMS-8040, Shimadzu, Kyoto, Japan) equipped with Inertsil® ODS-4 colon (3 µm, 2.1 x 50 mm, GL Sciences, Japan). Flow rate, colon temperature, and mobile phases A and B were 0.4 mL/min., 40 °C, and 0.2 mmol/L for ammonium formate + 0.004 % formic acid and methanol, respectively. Parent and daughter ions and retention time for Flx were 310.2, 148.1, and 8.033 min, respectively. Parent and daughter ions and retention time for NorFlx were 296.1, 134.1, and 8.011 min, respectively. As our device and method could not detect environmental concentrations, we spiked environmental solutions with 1 µg/L Flx or NorFlx and calculated the difference between 1 µg/L and spiked solutions. Method accuracy expressed in terms of recovery was ≥89.78 % for Flx and ≥93.00 % for NorFlx. r^2 values of standard calibration curve prepared for both chemicals was 0.999 (Table 1).

For the chronic toxicity experiments we used third-generation offspring younger than 24 h divided in seven groups and kept in beakers containing 800 mL of ASTM hard water: control group, Flx group (0.091 µg/L as environmentally realistic concentration), NorFlx group (0.011 µg/L as environmentally realistic concentration), Flx+NorFlx group (mixture of their environmentally realistic concentrations), 10 x Flx group (0.91 µg/L), 10 x NorFlx group (0.11 µg/L), and 10 x Flx+10 x NorFlx group (a mixture of their 10-fold environmental concentrations). Twenty-one animals were placed in each beaker, and four beakers for each group were used as replicates. The experiments lasted 21 days. Water was completely changed every third day. During the change, 1.5 mL/L of food was pipetted into beakers. Less than 24 h-old nestlings in each beaker were counted and collected into 50 mL Falcon tubes (containing 50 % ethyl alcohol) at 24 h intervals starting from the eighth day. Collected animals were stored at +4 °C before microscopy.

For subacute, four-day exposure experiments we used the fourth-generation offspring of 500 less than 24 h-old animals divided into two plastic 15 L containers filled with ASTM hard water. The animals were fed 1.5 mL/L trout chow/yeast/alfalfa mixture for 17 days. On day 17, 21 animals per group were placed into a beaker containing 800 mL of ASTM hard water. The groups included control, 100 x Flx (9.1 µg/L), 100 x NorFlx (1.1 µg/L), and 100 x Flx+100 x NorFlx (a mixture of their 100-fold environmental concentrations). Five beakers for each group were used as replicates. 1.5 mL/L of food was pipetted into each beaker. After 48 h, water was changed, and the animals stopped receiving food. At the end of 96 h exposure period, animals were 21 days old.

At the end of subacute and chronic experiments, one animal per beaker was placed into a microplate well and the microplate stored at -80 °C until 5,6-dichloro-2-[(E)-3-(5,6-dichloro-1,3-diethylbenzimidazol-3-ium-2-yl)prop-2-enylidene]-1,3-diethylbenzimidazole;iodide (JC-1; CAS

Table 1 Confirmation of water concentrations of fluoxetine (Flx) and norfluoxetine (NorFlx) after serial dilution

Concentrations	0.091 µg/L	0.91 µg/L	9.10 µg/L	0.011 µg/L	0.11 µg/L	1.10 µg/L
Flx	0.090±0.007	1.18±0.06	9.39±0.05	-	-	-
NorFlx	-	-	-	0.010±0.000	0.15±0.01	1.14±0.01

Data are given as means ± standard deviations (*N*=3)

No: 3520-43-2) staining for MMP analysis. The remaining animals from each beaker were carefully collected on a filter, washed with clean ASTM hard water, blotted dry, placed into one micro tube, weighed (all these steps were done on ice as much as possible), and stored at -80 °C until biochemical analysis. The procedure was repeated with four beakers as replicates until we acquired an adequate sample for Western blotting of cytochrome c and ERK1/2.

Before biochemical analysis, a sample of 20 animals from each microtube were homogenised with chilled 100 mmol/L KCl and 1 mmol/L EDTA (containing pH 7.4 100 mmol/L potassium phosphate buffer on ice at a ratio of 1:4 w/v in a homogeniser equipped with stainless steel shaft (HS30T, WiseStir, Daihan, Korea). The homogenate was then centrifuged at 10000 *g* and +4 °C for 10 min. The obtained supernatant was used to measure the activity of glutathione peroxidase (GPx) and cholinesterase (ChE) and to measure thiobarbituric acid-reactive substances (TBARS) and total proteins. Spectrophotometric analysis was done in a microplate reader (EON™, BioTek, Winooski, VT, USA).

Glutathione peroxidase (GPx) protects the cell against oxidative stress by reducing hydrogen peroxide (H_2O_2) and organic peroxides via glutathione consumption (20). It was analysed according to the method described by Beutler (21). Briefly, 25 µL of supernatant was incubated with 10 µL of 1 mol/L pH 8.0 Tris buffer, 25 µL of 0.1 mol/L glutathione (GSH), 100 µL of 10 U/mL glutathione reductase (GR), and 100 µL of 2 mmol/L reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) at +37 °C for 10 min. The reaction was started with the addition of 7 mmol/L *t*-butyl hydroperoxide. Absorbance change was measured at 340 nm for 2 min using the 6.22 L/mmol/cm extinction coefficient.

Cholinesterases (ChE) are proposed as biomarkers for apoptosis (and neurotoxicity) because of increased expression during apoptotic conditions (22). Cholinesterase activity was analysed according to the method of Ellman et al. (23). Briefly, 100 µL of supernatant were added to a 125 µL mixture of 0.1 mol/L potassium phosphate buffer (pH 7.2), 10 mmol/L 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and 10 mmol/L sodium bicarbonate and incubated at room temperature for 10 min. The reaction was started with the addition of 100 µL of acetylthiocholine iodide. Absorbance change was measured at 405 nm for 5 min using the 13.6 L/mmol/cm extinction coefficient.

TBARS is a universal measure of reactive oxygen species (ROS) damage on unsaturated fatty acids (24). Its levels were measured according to the method described by Wills (25). 25 µL of supernatant was mixed with 25 µL of 10 % trichloroacetic acid (TCA). After centrifugation at 500 *g* for 10 min, 37.5 µL of supernatant were mixed with 37.5 µL of 0.65 % thiobarbituric acid and incubated at 100 °C for 10 min. The absorbance was measured at 535 nm and converted to concentration values using a standard calibration curve prepared with 1,1'3,3'-tetra methoxy propane.

Total protein levels were measured with a modified Lowry method (26). 10 µL of supernatant were mixed with 45 µL of Lowry reagent and incubated at room temperature for 10 min. Then, 45 µL of Folin & Ciocâlteu's phenol reagent were added, and the mixture incubated at room temperature for another 30 min. The absorbance was measured at 750 nm and converted to concentration values using a standard calibration curve prepared with bovine serum albumin (BSA).

JC-1 probe was used as qualitative measure of change in mitochondrial membrane potential (27). Membrane depolarisation is a common biomarker of apoptotic cell death (28, 29). At low potential, the membrane turns fluorescent green, but at higher potential it turns fluorescent red. Each *D. magna* sample was incubated in its microplate well with 6 mmol/L JC-1 in dark at room temperature for 90 min. Fluorescence was measured by a confocal laser microscope (LSM700, Carl Zeiss, Oberkochen, Germany).

In healthy cells cytochrome c is found between mitochondrial membranes and is mainly responsible for oxidative phosphorylation. However, its release into the cytosol triggers the intrinsic pathway of apoptosis through the formation of mitochondrial permeability transition pore (MPTP) (30). The other two apoptosis-related proteins we studied – extracellular signal-regulated kinases 1 and 2 (ERK1/2) – act together to increase the activity of anti-apoptotic proteins or their transcription factors to prevent apoptosis. However, they also behave as an apoptotic factor through yet unknown mechanisms (31). For Western blotting of cytochrome c and ERK1/2, 20 *D. magna* animals were pooled from four beakers per group (totalling 80 animals) and homogenised as one replicate with radioimmunoprecipitation assay (RIPA) lysis buffer. Protein levels were measured following a modified Lowry method (26), in which samples were mixed with lithium dodecyl sulphate buffer at a ratio of 3:1 v/v and denatured at +100 °C for 10 min. 100 µg of protein-containing samples were loaded onto 10 % polyacrylamide gels. Protein bands were constituted for about 1 h and transferred on polyvinylidene fluoride (PVDF) membranes with an iBlot 2 transfer device (Invitrogen, Waltham, MA, USA). The transfer lasted 10 min. A signal enhancer solution was used to better visualise the bands. PVDF membrane was saturated with 5 % BSA to prevent non-specific binding. The membranes were then treated with primary antibodies (cytochrome c and ERK1/2, diluted to 1/1000 concentration) at +4 °C for one night. After washing with PBS-Tris solution (PBS-T), the membranes were treated with secondary antibodies (diluted to 1/6000 concentration, anti-rabbit) at room temperature for another hour. Re-washed with PBS-T solution, the membranes were then treated with a solution of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT) to visualise and take images of

the bands. Their intensity was measured with ImageJ v1.46r software (National Institutes of Health, Sacaton, AZ, USA).

For morphometric analysis nestlings were placed in a concave microscope slide, which contained lactic acid solution. They were photographed under a binocular microscope (BX53, Olympus, Tokyo, Japan) equipped with a Canon EOS1200D camera (Canon Inc., Tokyo, Japan) with 40x magnification. Carapace length was measured between the anterior head and caudal spine basement (32) using the Micam v. 2.0 imaging software (http://www.science4all.nl/?Microscopy_and_Photography). The caudal spine was not included in this measurement, as it constituted another toxicity parameter. Maximum carapace width was measured laterally from the maximum width of carapace ends.

Statistical analysis

Biochemical and morphometric data were analysed with the Statistical Package for Social Sciences for Windows, version 17 (SPSS, SPSS Inc., Chicago, IL, USA). Normality of data distribution was tested with the Kolmogorov-Smirnov test. For data that were not distributed normally we used the Kruskal-Wallis non-parametric test. If the Kruskal-Wallis test revealed significant difference, we further tested it with the Mann-Whitney *U* test. For normally distributed data we used one-way analysis of variance (ANOVA). Homogeneity of variance was tested with Levene's test. Duncan *post hoc* test was used for homogeneous subsets, while Tamhane's T2 was used for non-homogeneous subsets. Data are presented as means ± standard deviations, and differences are considered significant at $P<0.05$.

RESULTS AND DISCUSSION

Table 2 shows neurotoxic and oxidative effects of chronic and subacute exposure to Flx, NorFlx, and their combinations. Cholinesterase activity was particularly high at 100-fold environmental concentrations, but NorFlx at environmental concentration and at ten times that also increased ChE activity, while the increase with Flx and Flx+NorFlx was not statistically significant. Li and Tan (33) also reported a rise in ChE in *D. magna* by triazophos and chlorpyrifos and interpreted it as an adaptive *de novo* synthesis to maintain homeostasis via discontinuation of acetylcholine-induced ion channel activity (34).

Considering that ChE activity is increased by apoptosis inducers *in vitro* and *in vivo* (22, 35), ChE can serve as a biomarker of apoptosis. The observed increase in ChE activity in our study may be the consequence of increased acetylcholine release, which we did not measure, but which was also reported elsewhere (36) and may be related to the known effects of serotonin. Namely, serotonin increases intracellular Ca^{2+} levels (37) and, in turn, stimulates acetylcholine release (38). Furthermore, consecutive synergistic action of serotonin and acetylcholine may intensify the Ca^{2+} uptake (39, 40), which can increase oxidative stress (41), as evidenced by other studies (42, 43). Ca^{2+} at high concentrations can also cause mitochondrial damage. ROS production and consecutive induction of apoptosis can be triggered via net intracellular Ca^{2+} uptake or release from endoplasmic reticulum stores (41). According to Yardimci et al. (44), increased ChE activity may be an adaptive mechanism against acetylcholine-induced and Ca^{2+}-mediated ROS production, which they

Table 2 Effects of fluoxetine (Flx), norfluoxetine (NorFlx), and their combinations on GPx, ChE, TBARS, and total protein levels in *Daphnia magna*

	GPx (µmol/min/mg protein)	ChE (µmol/min/mg protein)	TBARS (nmol/mg protein)	Total proteins (mg/mL)
Chronic				
Control	0.047±0.010^{a}	0.010±0.003^{ab}	8.169±2.892^{a}	1.143±0.271^{a}
Flx	0.048±0.002^{a}	0.012±0.001^{bc}	8.413±0.999^{a}	0.972±0.038^{a}
NorFlx	0.051±0.004^{a}	0.014±0.001^{c}	11.670±1.347^{ab}	0.996±0.032^{a}
Flx+NorFlx	0.056±0.010^{ac}	0.012±0.002^{bc}	8.750±1.106^{a}	0.948±0.037^{a}
10 x Flx	0.086±0.003^{b}	0.014±0.001^{c}	16.354±3.315^{b}	0.909±0.028^{a}
10 x NorFlx	0.062±0.010^{c}	0.010±0.001^{ab}	21.840±6.003^{c}	0.966±0.125^{a}
10 x Flx+10 x NorFlx	0.050±0.005^{a}	0.007±0.002^{a}	15.600±3.283^{b}	1.029±0.098^{a}
Subacute*				
Control	0.063±0.005^{a}	0.016±0.003^{a}	10.635±3.731^{a}	0.681±0.107^{a}
100 x Flx	0.175±0.071^{bc}	0.058±0.020^{b}	67.963±32.735^{b}	0.365±0.048^{b}
100 x NorFlx	0.273±0.221^{b}	0.053±0.009^{b}	24.030±7.179^{c}	0.278±0.071^{b}
100 x Flx+100 x NorFlx	0.129±0.042^{c}	0.054±0.012^{b}	35.388±15.764^{bc}	0.297±0.069^{b}

Data are presented as means ± standard deviations. Data that do not share the same letters are significantly different ($P<0.05$; $N=4$; *$N=5$)

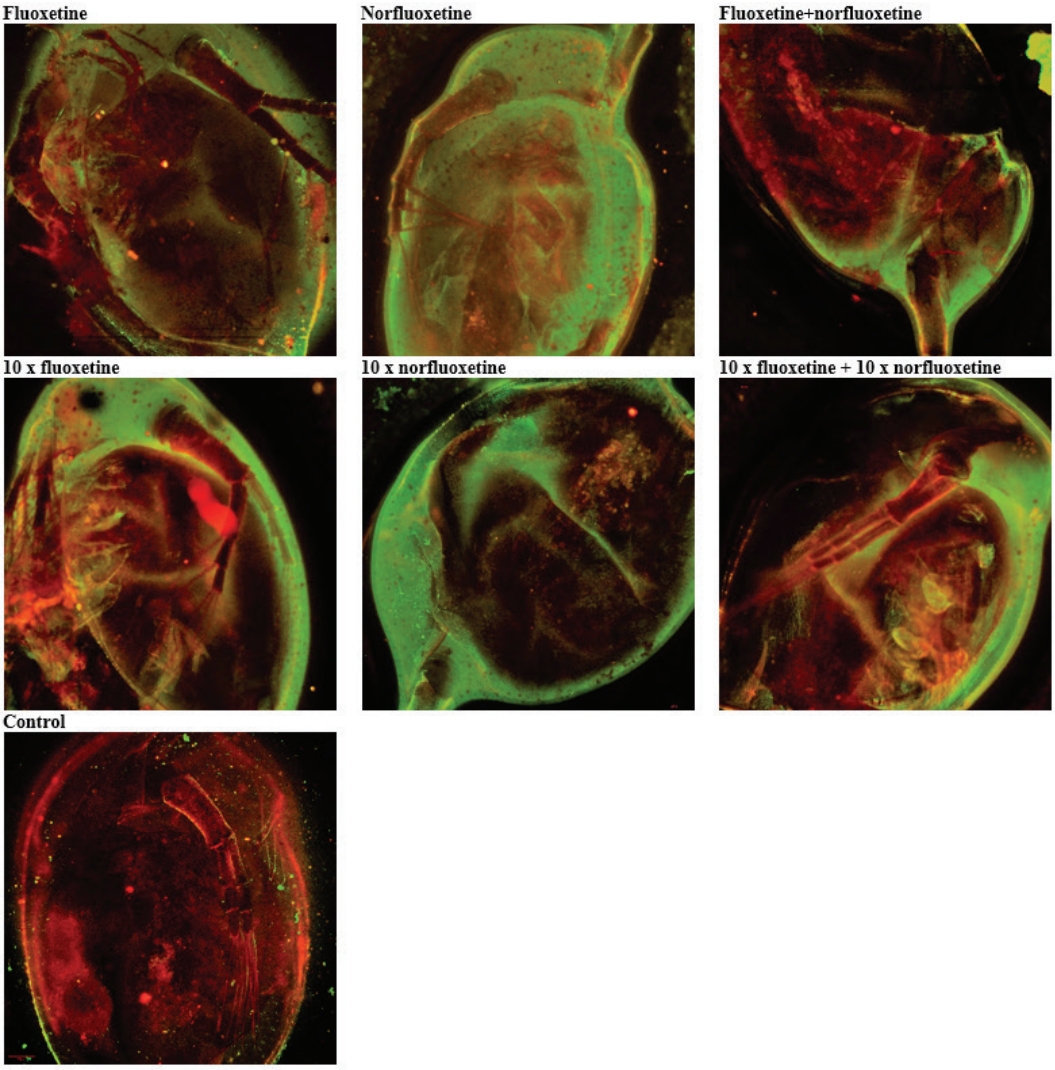

Figure 1 Chronic effects of fluoxetine, norfluoxetine, and their combinations at environmental or 10-fold environmental concentrations on mitochondrial membrane potential in *Daphnia magna* (200 μm)

observed in the kidney of imidacloprid-exposed rats. As for the oxidative effect observed in our study through higher lipid peroxidation levels at Flx and NorFlx concentrations higher than environmental, Yang & Dettbarn (45) reported a connection between high lipid peroxidation and increased acetylcholine levels caused by anticholinesterase effects of diisopropyl fluorophosphate.

At 100-fold environmental concentrations Flx, NorFlx, and their combinations decreased total protein levels, but this effect was not observed in chronic toxicity experiments involving environmental and 10-fold environmental concentrations (Table 2). Concentration- and duration-dependent decrease in total protein levels was also observed in *D. magna* exposed to herbicide tebuconazole (46). Selective serotonin re-uptake inhibitors (SSRIs) increase aerobic metabolism with oxygen consumption (47), which points to increased ROS production (29) and suggests that increased protein catabolism to compensate for increased energy demands may be an important biomarker of toxic stress, as suggested in a study by Villarroel et al. (48).

In contrast to our findings, Campos et al. (47) reported no significant changes in total protein and lipid content in adult *D. magna* exposed to much higher Flx concentrations (80 μg/L) for 24 h. Instead, they reported increased carbohydrate and oxygen consumption, which may point to higher ROS levels associated with increased aerobic metabolism (29). As several studies suggest higher protein carbonyl levels in oxidative stress (49), which ends with proteasomal degradation of oxidised proteins (50), the lowering of total protein levels in our subacute experiment may display the oxidative damage on tissue proteins.

Glutathione peroxidase as first-line defence against oxidative stress also increased at both 10 and 100 times Flx and NorFlx environmental concentrations. At environmental levels, however, its basal activity seems to have been sufficient to counter ROS and prevent oxidative damage (Table 2). A case in point could by the anticholinesterase insecticide chlorpyrifos study (49), which increased cytosolic ChE and GPx activities and H_2O_2 levels but not lipid peroxidation. In our study, the concentration-

Table 3 Chronic effects of fluoxetine (Flx), norfluoxetine (NorFlx), and their combinations at environmental or 10-fold environmental concentrations on carapace length, maximum carapace width, and caudal spine length in less than 24 h-old *D. magna* offspring born in experimental beakers

	Carapace length (µm)	Max. carapace width (µm)	Caudal spine length (µm)	Carapace length (µm)	Max. carapace width (µm)	Caudal spine length (µm)	Carapace length (µm)	Max. carapace width (µm)	Caudal spine length (µm)
	1st generation offspring			2nd generation offspring			3rd generation offspring		
Control	777.6±50.4	430.0±41.0	403.2±30.4	880.9±85.9^{a}	473.7±86.9^{acd}	415.3±31.5	847.0±46.1^{a}	490.6±52.8^{ad}	411.5±42.8^{a}
Flx	773.0±37.3	414.5±43.1	385.2±26.4	827.8±76.1^{b}	437.5±62.9^{c}	400.6±33.1	806.9±45.7^{b}	475.6±53.6^{ac}	371.2±42.0^{b}
NorFlx	748.5±30.4	408.5±38.9	354.0±34.6	842.3±72.5^{b}	448.5±66.1^{ac}	403.5±28.6	838.7±41.8^{a}	475.0±38.9^{ac}	378.1±44.4^{bc}
Flx+NorFlx	774.2±33.9	420.9±47.8	367.0±29.3	857.3±56.1^{a}	466.0±55.1^{ad}	397.4±32.3	812.4±45.2^{b}	469.2±45.4^{bc}	369.7±39.9^{b}
10 x Flx	742.6±32.7	400.9±45.8	345.1±25.9	874.1±107.5^{ab}	496.4±97.7^{de}	406.1±43.0	906.2±80.9^{c}	524.4±70.2^{e}	397.3±40.2^{ac}
10 x NorFlx	764.2±34.6	407.6±36.9	358.8±26.2	893.8±101.5^{ac}	521.6±78.7^{be}	401.4±41.7	857.9±69.4^{a}	492.8±68.0^{abf}	379.9±41.5^{bc}
10 x Flx+10 x NorFlx	733.7±34.1	388.5±43.2	349.4±41.9	925.6±102.1^{c}	544.5±81.9^{b}	408.0±53.6	915.7±105.3^{c}	524.0±90.6^{def}	380.3±56.7^{bc}
	4th generation offspring			5th generation offspring			Average		
Control	887.6±22.5^{a}	483.0±31.2^{a}	392.0±31.3^{ae}	899.7±64.5^{a}	497.7±49.2^{a}	385.8±30.3^{ac}	865.0±70.3^{a}	478.3±59.6^{a}	401.0±35.4^{a}
Flx	842.9±27.4^{bd}	456.7±33.7^{bc}	414.1±32.4^{b}	783.7±58.2^{b}	446.4±35.1^{b}	363.0±51.7^{a}	805.9±58.5^{b}	444.5±50.0^{b}	386.8±42.6^{b}
NorFlx	852.2±55.1^{bc}	449.7±52.4^{b}	372.0±28.7^{cd}	890.9±100.1^{c}	503.9±71.5^{af}	389.8±49.7^{cb}	831.1±78.7^{c}	455.8±62.4^{bd}	378.8±41.5^{c}
Flx+NorFlx	878.8±56.8^{a}	473.4±60.4^{ac}	367.7±31.6^{d}	891.2±94.0^{ac}	532.3±63.7^{c}	395.5±35.5^{cb}	836.7±74.8^{c}	468.3±65.8^{cef}	378.7±36.0^{c}
10 x Flx	845.3±47.1^{bd}	454.4±37.5^{b}	395.9±29.0^{a}	836.9±35.9^{d}	462.1±30.5^{ed}	394.0±25.7^{b}	841.0±86.0^{c}	467.6±73.6^{deg}	387.9±39.6^{bd}
10 x NorFlx	855.9±22.2^{c}	480.4±24.8^{a}	394.0±41.5^{a}	838.4±98.5^{e}	476.5±69.7^{def}	386.7±44.6^{ab}	837.8±84.4^{c}	472.2±71.4^{afg}	382.3±41.5^{cd}
10 x Flx+10 x NorFlx	833.0±42.3^{d}	465.9±25.9^{c}	381.0±23.8^{ce}	833.0±88.7^{de}	470.2±61.0^{be}	381.0±45.3^{ab}	849.9±105.7^{c}	480.2±84.1^{afg}	381.0±48.9^{cd}

Data were presented as means ± standard deviations. There is a statistical difference between the data that does not share the same letters (P<0.05; N=9-27; for average calculation N=227-267)

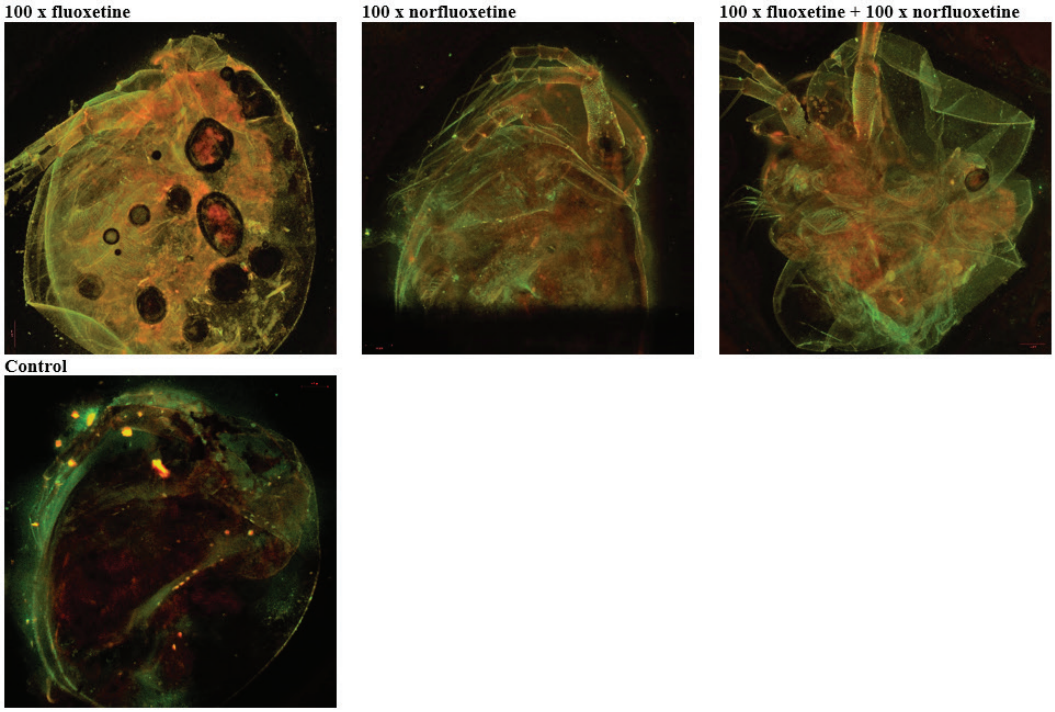

Figure 2 Effects of fluoxetine, norfluoxetine, and their combinations at subacute, 100-fold environmental concentrations on mitochondrial membrane potential in *Daphnia magna* (200 µm)

dependent increase in GPx activity and lipid peroxidation levels should be viewed through redox balance, which tipped toward oxidation at higher Flx and NorFlx concentrations. Similar concentration-dependent changes in *D. magna* was reported with organochlorine insecticide endosulfan (51) and antibiotic chloramphenicol (52).

Mitochondrial membrane depolarisation is a common marker of cell death and physiological condition of cells and tissues (28, 53). In our study, mitochondrial membrane depolarisation was observed in all the Flx, NorFlx, and combination groups in both chronic and subacute exposure (Figures 1 and 2) and especially with NorFlx. This suggests that Flx and NorFlx exert their toxicity via mitochondrial mechanisms directly or indirectly. Similar mechanisms have been observed in other studies in different models, including *D. magna* (42, 54, 55).

Mitochondrial membrane depolarisation is an important mechanism for the release of cytochrome c from the mitochondria (28), and cytochrome c release induces mitochondrial apoptosis in mammalian cells (56). In our study, cytochrome c protein levels were generally increased, except for the Flx and 10 x Flx+10 x NorFlx combination groups (Figures 3 and 4). However, increased cytochrome c protein content alone does not provide strong evidence of the mitochondrial release and apoptosis in our study, but in combination with increased lipid peroxidation and mitochondrial membrane depolarisation it does give grounds for such an assumption. We believe that increased cytochrome c levels in our study are related to intensive stress conditions and higher mitochondrial damage induced by Flx and NorFlx. Such a result was also found in the liver of mercury chloride-exposed rats (57).

Table 4 Chronic effects of fluoxetine, norfluoxetine, and their combinations at environmental or 10-fold environmental concentrations on brood production of *D. magna*

	1st generation offspring	2nd generation offspring	3rd generation offspring	4th generation offspring	5th generation offspring	Average
Control	76.8±13.6^{a}	50.1±16.5^{ac}	44.1±16.4^{a}	73.9±15.3^{a}	84.8±32.3^{ac}	65.9±25.1^{a}
Flx	79.8±24.4^{ac}	46.2±5.7^{a}	62.7±18.1^{b}	78.7±32.1^{a}	81.3±29.7^{a}	69.7±26.9^{a}
NorFlx	143.3±44.7^{b}	69.5±25.4^{cdf}	140.7±51.8^{c}	135.6±64.2^{bc}	62.2±22.8^{cd}	110.3±56.6^{b}
Flx+NorFlx	126.3±29.9^{b}	94.3±43.4^{bd}	144.3±29.5^{c}	135.3±68.1^{b}	45.4±9.3^{b}	109.1±53.7^{b}
10 x Flx	129.5±59.0^{bd}	53.5±29.2^{aeg}	60.6±9.3^{b}	84.9±58.6^{ac}	41.8±17.4^{bd}	74.1±48.0^{ac}
10 x NorFlx	70.6±21.9^{a}	42.3±26.8^{e}	57.7±29.9^{ab}	52.7±33.2^{a}	67.6±27.6^{c}	58.2±29.1^{c}
10 x Flx + 10 x NorFlx	98.6±31.8^{cd}	64.4±20.0^{fg}	56.8±8.9^{b}	60.6±48.5^{a}	42.3±15.5^{b}	64.5±33.4^{ac}

Data were presented as means ± standard deviations. There is a statistical difference between the data that does not share the same letters (P<0.05)

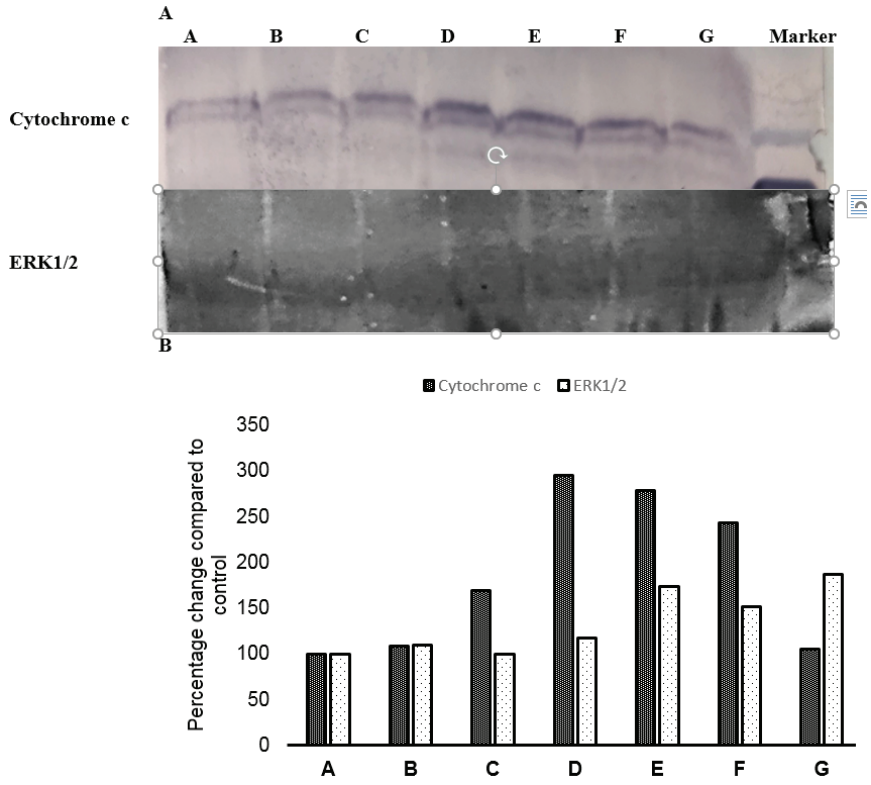

Figure 3 Chronic effects of fluoxetine, norfluoxetine, and their combinations at environmental or 10-fold environmental concentrations on cytochrome c and ERK1/2 protein levels in *Daphnia magna.* A – control; B – fluoxetine; C – norfluoxetine; D – fluoxetine+norfluoxetine; E – 10 x fluoxetine; F – 10 x norfluoxetine; G – 10 x fluoxetine+10 x norfluoxetine

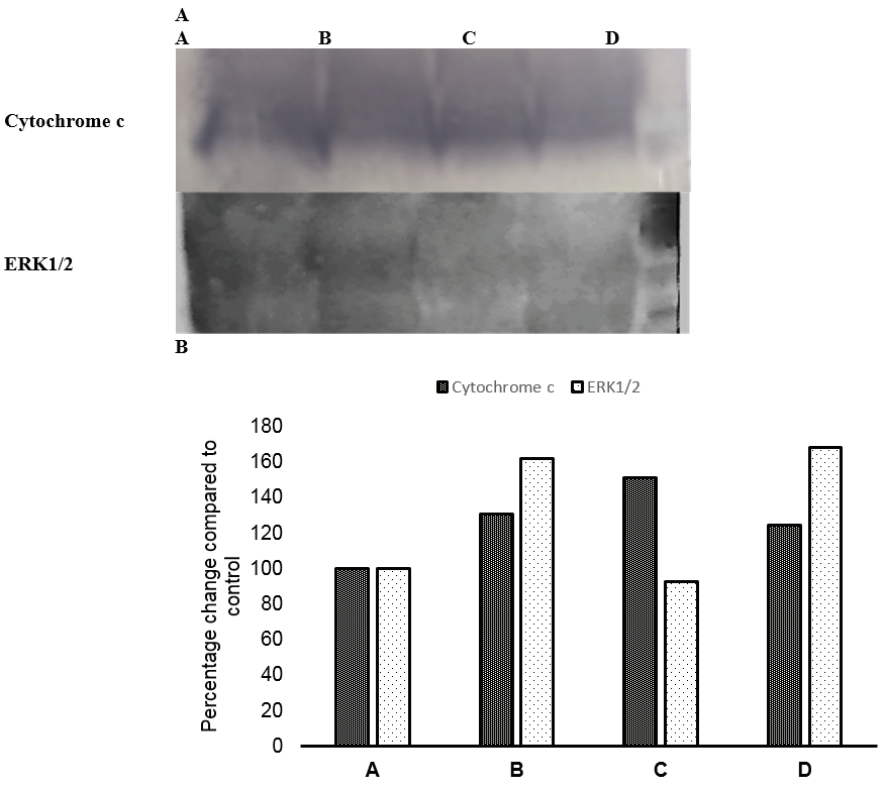

Figure 4 Subacute effects of fluoxetine, norfluoxetine, and their combinations at 100-fold environmental concentrations on cytochrome c and ERK1/2 protein levels in *Daphnia magna*. A – control; B – 100 x fluoxetine; C – 100 x norfluoxetine; D – 100 x fluoxetine+100 x norfluoxetine

Either compound or their combination did not affect ERK1/2 protein levels at environmental concentrations but did at higher concentrations, except for 100 times higher NorFlx alone (Figures 3 and 4). ERK1/2 is activated by a toxicant and regulates response to cellular damage (58). It promotes cell survival against apoptotic mechanisms, but in certain condition it also favours apoptosis (31). This may explain different responses to NorFlx in our study and calls for further investigation. We believe that increase in ERK1/2 protein levels may be an adaptive response to apoptotic conditions.

Table 3 shows a significant increase in carapace length and width in the 2nd and 3rd generation offspring of the 10 x Flx+10 x NorFlx and 10 x Flx groups, respectively. Width also increased in the 10 x NorFlx group. Prey animals experience a variety of changes to avoid their predators. They may include toxin production (physiological changes), late breeding (life-cycle changes), and production of spicules and sheathing (morphological changes) (59). Flx alone and in combination with its metabolite may have triggered this avoidance behaviour at 10-fold environmental concentrations. Decreased carapace length, width, and spine length seen in other groups since the 2nd generation offspring may be related to lower energy production, as reported elsewhere (60, 61). In a study by Pery et al. (62) Flx also decreased carapace length of less than 24 h-old 3rd generation *D. magna*, but the effect was observed at higher concentrations than those used in our study (62). Campos et al. (63) suggest that decreased body size in offspring is a result of poor adaptation to toxic conditions and we believe that long-term exposure to SSRIs may impair adaptation of non-target populations even at low concentrations.

NorFlx alone and in combination with Flx significantly increased brood production at their environmental concentrations until the 5th generation offspring (Table 4). A similar increase in the first four generations was also observed with Flx and its combination with NorFlx at 10-fold concentrations. Campos et al. (47) suggested that brood production of *D. magna* in their study may have been increased due to higher serotonin availability. Serotonin affects the neurohormones, which play a role in reproduction, growth, pubescence, larval development, immune functions, metabolism, behaviour, and colour physiology in crustaceans (64). We therefore believe that Flx alone or in combination with NorFlx may have increased brood rate by blocking serotonin reuptake in our study.

However, in the 5th generation offspring brood production dropped in all groups (Table 4), which may be related to the bioaccumulation of Flx and NorFlx in *D. magna* (14). A similar drop in brood production rate was seen at higher Flx concentrations and longer exposure in other studies (62, 63). In fish models higher Flx and NorFlx concentrations were reported to decrease egg production (3, 65).

In conclusion, *D. magna* can serve as a mechanistic model for understanding toxic effects in non-target organisms. However, more studies are needed to explain their cellular mechanisms as clearly as those of the vertebrates. Flx, NorFlx, and their combinations induced morphological and/or biochemical responses, especially at 10-fold and 100-fold environmental concentrations, which suggests that their increasing presence in aquatic environments will have even worse effects on non-target organisms than they have now. However, our study has left some questions unanswered, and future research of serotonin, acetylcholine, Ca^{2+}, and ROS levels should address them, including the mechanistic relationships between them and the parameters studied here.

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Conflicts of interest

None to declare.

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Oksidacijski i apoptotski učinci fluoksetina i njegova metabolita norfluoksetina u vodenbuhe *Daphnia magna*

Cilj je ovoga istraživanja bio utvrditi oksidacijski i apoptotski potencijal fluoksetina, antidepresiva raširenoga u Turskoj i svijetu, i njegova metabolita norfluoksetina na modelu vodenbuhe *Daphnia magna* koji nije ciljani organizam djelovanja spojeva. Također smo željeli vidjeti kako izloženost toj skupini antidepresiva (specifičnih inhibitora ponovne pohrane serotonina) može utjecati na vodeni okoliš u kojem oni završe. Mlade jedinke vodenbuhe bile su izložene fluoksetinu (0,091 µg/L) i norfluoksetinu (0,011 µg/L), odvojeno i u kombinaciji, pri koncentracijama zamijećenima u okolišu i deseterostrukim okolišnim koncentracijama u trajanju od 21 dan (kronična izloženost). Jedna je skupina 17 dana starih vodenbuha također bila izložena stostrukoj okolišnoj koncentraciji u trajanju od četiri dana (subakutna izloženost). Potom su u životinja spektrofotometrijom izmjerene aktivnosti enzima glutation peroksidaze (GPx) i kolinesteraza (ChE) te razine reaktivnih tvari tiobarbituratne kiseline (TBARS) i ukupnih proteina. Potencijal mitohondrijske membrane (MMP) utvrđen je fluorescencijom, a proteini citokrom c i ERK1/2 Western blot metodom. Ovo je prvi put da su se u vodenbuhi citokrom c i ERK1/2 utvrđivali na razini proteina. Također je mikroskopski izmjerena dužina i širina oklopa i dužina repnog dijela kralježnice vodenbuha. Pri okolišnim koncentracijama fluoksetin i norfluoksetin doveli su do povišene aktivnost ChE i većeg razmnožavanja te smanjenja (dužine i širine) karapaksa i repne bodlje u podmlatka i depolarizacije mitohondrijske membrane. Pri deseterostrukim okolišnim koncentracijama porasle su razine aktivnosti GPx, lipidne peroksidacije, citokroma c i ERK1/2 proteina. Norfluoksetin je pritom iskazao najsnažnije djelovanje te doveo do pada razmnožavanja. Slični su učinci primijećeni kod subakutne izloženosti stostrukim okolišnim koncentracijama fluoksetina i norfluoksetina, osim što je ona dovela i do pada ukupnih proteina. Naši rezultati potvrđuju da fluoksetin i norfluoksetin imaju oksidacijski i apoptotski učinak u vodenbuhe. Taj životinjski model može odlično poslužiti za stjecanje uvida u mehanizme toksičnoga djelovanja tvari u okolišu, no potrebna su daljnja istraživanja prije nego što ti mehanizmi postanu potpuno jasni.

KLJUČNE RIJEČI: citokrom c; ERK1/2; GPx; kolinesteraza; lipidna peroksidacija; oksidacijski stres; potencijal mitohondrijske membrane; TBARS