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# Antioxidant responses and DNA damage in primary hepatocytes of Van fish (*Alburnus tarichi*, Güldenstadt 1814) exposed to nonylphenol or octylphenol

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**ABSTRACT**

Alkylphenols, a nonionic surface-active agent group, such as nonylphenol (NP) and octylphenol (OP) are important endocrine-disrupting chemicals (EDC). In this study, the dose- and time-dependent effects of NP and OP were investigated in the primary hepatocyte culture of Van Fish. In this study, samples were taken at different times and biochemical parameters were studied separately. The effects of the chemicals used on SOD, CAT, GSH-Px, MDA, and 8-OHdG were investigated in hepatocyte culture. The antioxidants SOD and CAT were observed to increase in all groups in the primary hepatocyte cultures at the 24th hour after NP and OP administration, whereas the GSH-Px level was observed to increase with OP at the 24th hour and with NP at the 48th hour. The MDA level was observed to reach its highest value for both chemicals in the 24th hour, and the 8-OHdG level was observed to increase toward the end of the follow-up time, compared to the control group ($p < 0.05$). In conclusion, different doses of NP and OP were found to induce an increase in the levels of antioxidants and the MDA level in Van Fish primary hepatocyte culture. DNA damage, on the other hand, may be considered to appear after longer-term exposure to NP and OP.

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**KEYWORDS**
Oxidative stress; DNA damage; MDA; Van fish; antioxidant

## Introduction

Toxic substances are known to cause free radical formation, and free radicals are known to play a role in many diseases (Parks et al. 1995, Takoudes and Haddad 1999). Many chemicals released into the environment impair biochemical and physiological processes, and scientific studies indicating the negative effects of these substances on the health of humans and other living organisms are rapidly increasing in number (Solomon and Schettler 2000, Ishihara et al. 2007).

Endocrine-disrupting chemicals (EDCs) behave like hormones during metabolism. Thus, these chemicals, referred to as endocrine substances, lead to cellular and even hereditary damage in living organisms including humans, animals, and plants (Norris and Carr 2013). They may be naturally present in nature and are also used as components of many synthetic and industrial products (Bigsby et al. 1999, Lee 2007). These substances are not completely eliminated during wastewater processing and accumulate within the ecosystem (Kümmerer 2004). Due to their widespread use, they have even been reported to be present in snow and rainwater (Fries and Puttmannb 2004, Vethaak et al. 2005).

Environmental endocrine substances mainly include diethylstilbestrol, dichlor diphenyl trichloroethane (DDT), polychlorobiphenyls (PCB), and alkylphenol ethoxylates (APE). Nonylphenol (NP) and octylphenol (OP), which are the most frequently studied members of the APE compounds, are estrogenic, carcinogenic, and toxic chemical substances of the xenoestrogen group (Nimrod and Benson 1996, Hughes et al. 2000). APE compounds are toxic agents that are widely used in many industrial fields as detergents, herbicides, insecticides, dyes, cosmetics, plastics, surface-active substances, gas oil, and aviation type fuel (Veld et al. 2006). NP and OP are produced via the hydrolytic removal of the ethoxylate groups from APEs (Nimrod and Benson 1996).

Like other toxic substances, NP and OP are identified as sources of oxidative stress (Kehrer 1993, Okai et al. 2004). The antioxidant defense systems are present in living organisms in order to control free radical formation and to prevent the harmful effects of these molecules. However, in certain circumstances, the antioxidant defense system cannot eliminate the harmful free radicals completely and a situation called oxidative stress occurs. This leads to damage to the lipids, proteins and DNA structure of the organism (Pham-Huy et al. 2008). Substances called antioxidants act to fight against oxidative stress by reacting with the radicals and preventing the progression of auto-oxidation and peroxidation (Fang et al. 2002, Halliwell and Gutteridge 2007). Antioxidant defense has different mechanisms such as preventing the production of radical metabolites, scavenging the produced radicals, repairing cellular damage, stopping reaction chains of radical formation, and increasing the endogenous antioxidant capacity (Gutteridge 1995, Halliwell and Gutteridge 2007).

Toxic chemicals may cause an increase in fish 8-OHDG activity and changes in oxidative stress parameters (Alak et al. 2017, Topal et al. 2017). Studies have shown gonadal abnormalities in both male and female Van Fish from Lake Van (Oğuz and Yeltekin 2014), which may be related to endocrine disrupters due to chemical contamination of the lake (Ünal et al. 2007).

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Van Fish is important because it is the only fish species in Van Lake which has pH 9–10 (Kipfer 1994) and the fish are an important protein source with economic value in the region. Herein, the effects of the endocrine disrupters NP and OP, at $10^{-6}$, $10^{-5}$, $10^{-4}$ molar concentrations (2nd, 4th, 6th, 12th, 24th, and 48th hours) on MDA (malondialdehyde) and DNA (8-hydroxy-2-deoxyguanosine (8-OHdG)) damage and the antioxidant defense system (superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT)) were investigated in the primary hepatocyte culture of Van Fish. For this reason, the aim is to investigate the effects of NP and OP on the antioxidant system and in the hepatocyte culture of Van Fish.

## Materials and methods

### Fish

The fish to be used for the primary hepatocyte culture were caught via casting nets from water sources entering the lake. In this study, Van Fish aged 2–3 years, weighing 80–90 g were used. The Van Fish taken into the laboratory were used for cell culture following an adaptation period of 1 week. Permission was granted for the study by the Ministry of Food, Agriculture and Livestock and Van Yuzuncu Yil University Animal Research Local Ethics Committee.

### Hepatocyte isolation

The two-stage perfusion method modified from mammalian hepatocyte isolation was used to isolate the hepatocytes of adult male Van Fish (Berry and Friend 1969). After the fish were anesthetized with aminobenzonate methanesulfonate (MS 222, 100 mg/L), they were sterilized by immersion in 70% ethanol. The fish were then dissected in laminar flow under sterile conditions and the liver was carefully excised and placed into a watch glass. The fat tissue on the liver was removed. The liver tissue was divided into small pieces and kept in sterile buffer (hepatocyte buffer: 0.145 M NaCl, 5.4 mM KCl, 5 mM EDTA, 1.1 mM $KH_2PO_4$, 12 mM $NaH_2CO_3$, 3 mM $NaHPO_4$, pH 7.6) at room temperature. This process was repeated several times within 30 minutes. The tissue was then transferred into hepatocyte buffer containing 2.5 mM $CaCl_2$ without EDTA. Hence, the tissue cleaned of blood was incubated in Leibovitz-15 (L-15) containing collagenase (0.5 mg/ml) for 30 minutes. After the incubation, the cells were mechanically agitated with a plastic transfer pipette. The cells obtained at the end of this process were centrifuged a few times at $90 \times g$ for 5 minutes until the supernatant in the suspension became clear, and L-15 replaced the supernatant. The cell pellet was suspended by mechanical agitation. Antibiotic–antimycotic solution (10 µl/ml) and 5 mM $NaHCO_3$ were added to the medium to prevent contamination and for pH optimization (pH: 7.6). Isolated cells were stained with trypan blue (4%), and the live and dead cell counts were performed using the Thoma slide. The rate of live cells in the culture, to which attention was paid, was over 90%. Erythrocytes were observed in culture after the process of isolation. However, the rate of erythrocytes never exceeded 10% (Pärt et al. 1993, Wan et al. 2004).

### Cell culture

In the present study, six adult male fish were used. Isolated hepatocytes were seeded at a density of $1 \times 10^6$ cells/ml in 24-well culture plates (Greiner, France), which had previously been coated with poly-L-lysine. In total, 1000 µl medium was added to each well, and the cultures were then incubated at 18 °C in humid atmosphere. The morphological changes and the contamination check were investigated using an inverted microscope (DM 6000 Leica Microsystems, Wetzlar, Germany) and photographs were taken. The pH changes in the culture medium were checked regularly. NP and OP were dissolved in ethanol. At the end of the culture time, 1000 µl NP and OP ($1 \times 10^{-6}$, $1 \times 10^{-5}$, and $1 \times 10^{-4}$ M) were added to the medium and only solvent was added to the control group. To prevent the toxic effects of ethanol, the final concentrations in the culture media never exceeded 0.1%. Each of the chemicals and the control group were added to the three wells. The culture media were collected from plates and kept in the deep freeze at −80 °C until measurements for biochemical analysis.

### Biochemical analyses

Samples were taken at different times (2, 4, 6, 12, 24, and 48 h) and biochemical parameters were studied separately. The activity of the SOD enzyme was measured using the Randox-Ransod enzyme kit at 505 nm and 37 °C with a UV spectrophotometer (Shimadzu UV-1201, UV-Vis Japan) (Flohe and Ötting, 1984, Xia et al. 1995). The reaction catalyzed by xanthine oxidase generates uric acid and superoxide radicals from xanthine. Superoxide radicals form a red-colored farmazon compound when treated with inositol (INT). SOD activity is measured by the degree of inhibition of this reaction. The activity of CAT enzyme was measured using the UV spectrophotometer method of Aebi (1984) at 240 nm based on hydrogen peroxide ($H_2O_2$) catalase destruction. The reaction mixture contained potassium phosphate buffer and $H_2O_2$. The level of MDA was determined using the method of Placer et al. (1966) via UV spectrophotometer measurements of pink color absorbance at 532 nm, observed as a result of thiobarbituric acid (TBA) and MDA reactions. GSH-Px enzyme activity was measured using the Randox-Ransod enzyme kit at 340 nm and 37 °C under UV spectrophotometer (Paglia and Valentine 1967, Flohe and Gunzler 1984). The GSH-Px activity was calculated from the decrease in absorbance values. For the detection of liver 8-hydroxy-20-deoxyguanosine (8-OHdG) activity, a competitive ELISA for 8-OHdG analysis kit (Catalogue No.: 201-00-0041) (SunRed) was used according to the manufacture's protocol (Fish (8-OHdG) ELISA kit, DAS, Roma, Italy). The study was carried out at room temperature.

### Statistical analysis

The results are expressed as mean ± standard error. The one-way analysis of variance (ANOVA) and Duncan's multiple

comparison tests were applied to the data, and the differences between the data were determined ($p < 0.05$). SPSS 23 software was used for statistical calculations.

## Results

### *Effects of NP and OP exposure on antioxidant enzyme level*

The effect of OP on SOD activity of hepatocyte culture is shown in Figure 1. Generally, at 24 hours, the SOD level in all groups was significantly higher than the other times. At 48 hours, the SOD level again decreased significantly ($p < 0.05$). The effect of NP on SOD activity of hepatocyte culture is shown in Figure 2. For NP, SOD reached the highest level at 24 hours with a significant increase. The SOD concentration in all groups also showed a significant decrease at 48 hours ($p < 0.05$).

The effect of OP on CAT activity of hepatocyte culture is shown in Figure 3. The CAT level increased significantly at all hours for the OP4 ($10^{-4}$ M) concentration. The CAT level, which was high in the 24th hour, decreased significantly in the 48th hour ($p < 0.05$). The effect of NP on CAT activity of hepatocyte culture is shown in Figure 4. The CAT level increased significantly at all hours with NP5 ($10^{-5}$ M) concentration ($p < 0.05$). The CAT level decreased again at 48 hours at all concentrations ($p < 0.05$).

The effect of OP on GSH-Px activity of hepatocyte culture is shown in Figure 5. The GSH-Px level was significantly increased at 48 hours at all concentrations ($p < 0.05$). The effect of NP on GSH-Px activity of hepatocyte culture is shown in Figure 6. The GSH-Px level increased significantly at 24 hours compared to other times ($p < 0.05$).

### *Effects of NP and OP exposure on MDA level*

The effect of OP on MDA level of hepatocyte culture is shown in Figure 7. It was observed that MDA level showed a statistically significant increase with OP, especially at 24 and 48 hours. There was also a continuous significant increase for OP4 ($10^{-4}$ M) concentration ($p < 0.05$). The effect of NP on MDA level of hepatocyte culture is shown in Figure 8. The effect of NP on MDA level was seen at significant levels at the 24th and 48th hours. Again, the level of MDA with OP4 concentration was significantly increased in each group ($p < 0.05$).

### *Effects of NP and OP exposure on 8-OHdG level*

The effect of OP on 8-OHDG level of hepatocyte culture is shown in Figure 9. The effect of OP on 8-OHDG level shows a significant difference at the 24th and 48th hours. In general, there was also a significant increase for OP4 ($10^{-4}$ M) concentration ($p < 0.05$). The effect of NP on 8-OHDG level of hepatocyte culture is shown in Figure 10. The effect of NP on 8-OHDG level shows a significant difference at the 24th and 48th hours ($p < 0.05$).

## Discussion

EDC have been shown to have a widespread distribution in the natural environment and negative effects on living organisms (Colborn et al. 1996). Chemicals that alter the endocrine system may mimic, block, or change the activities of chemicals synthesized by the system itself. They particularly affect the thyroid gland, urinary system, and many other organs (Metzler and Pfeiffer 2001). Alkylphenols, a type of nonionic surface-active molecule, are the most important chemicals that impair the endocrine system. The break-up products of alkylphenols are nonylphenol and octylphenol with a wide range of use. These molecules have been demonstrated to reach dangerous environmental concentrations in recent studies (Colborn et al. 1996, Kelce and Wilson 2001, Metzler and Pfeiffer 2001). In a study conducted on wastewaters in the United States, the NP to OP proportion was 2–6 µg/L. The total APE level was within the 1350 to 11.00 µg/L range (Ruthann et al. 1998). Likewise, in Portugal, the APE levels in winter, spring, summer, and autumn were found to be

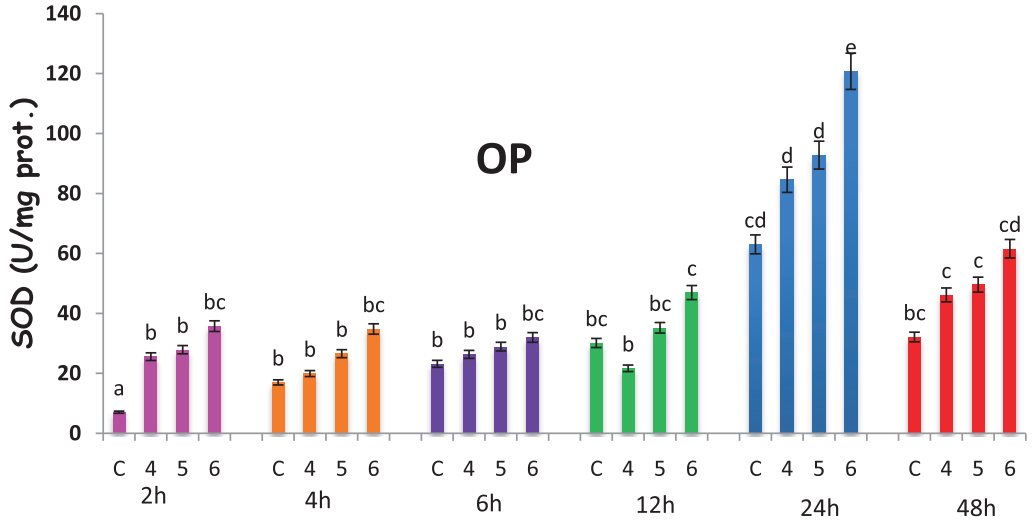



Figure 1. Time- and dose-dependent change in the SOD level in OP-applied hepatocyte culture (4, 5, 6: octylphenol $10^{-4}$, $10^{-5}$, $10^{-6}$ M). The difference between the columns with different letters is significant ($p < 0.05$) (The statistics letters show the differences between the concentrations).

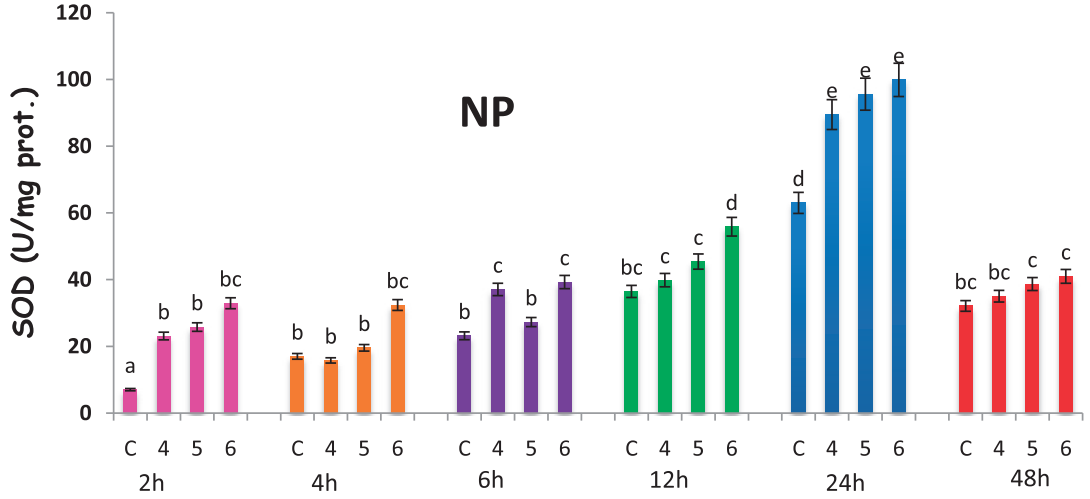



Figure 2. Time- and dose-dependent change in the SOD level in NP-applied hepatocyte culture (4, 5, 6: nonylphenol $10^{-4}$, $10^{-5}$, $10^{-6}$ M). The difference between the columns with different letters is significant ($p < 0.05$) (The statistics letters show the differences between the concentrations).

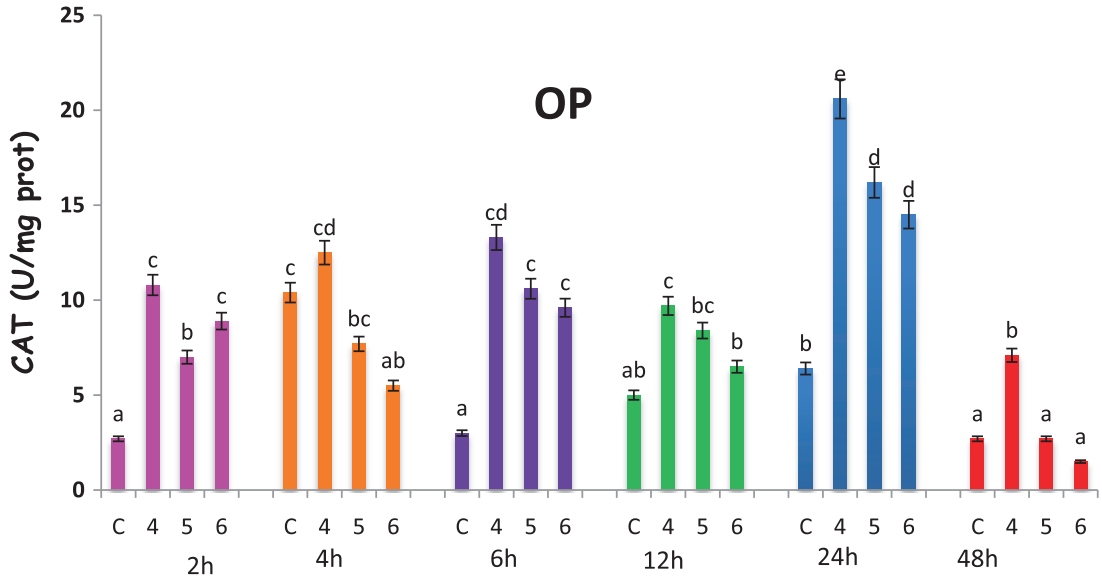



Figure 3. Time- and dose-dependent change in the CAT level in OP-applied hepatocyte culture (4, 5, 6: octylphenol $10^{-4}$, $10^{-5}$, $10^{-6}$ M). The difference between the columns with different letters is significant ($p < 0.05$) (The statistics letters show the differences between the concentrations).

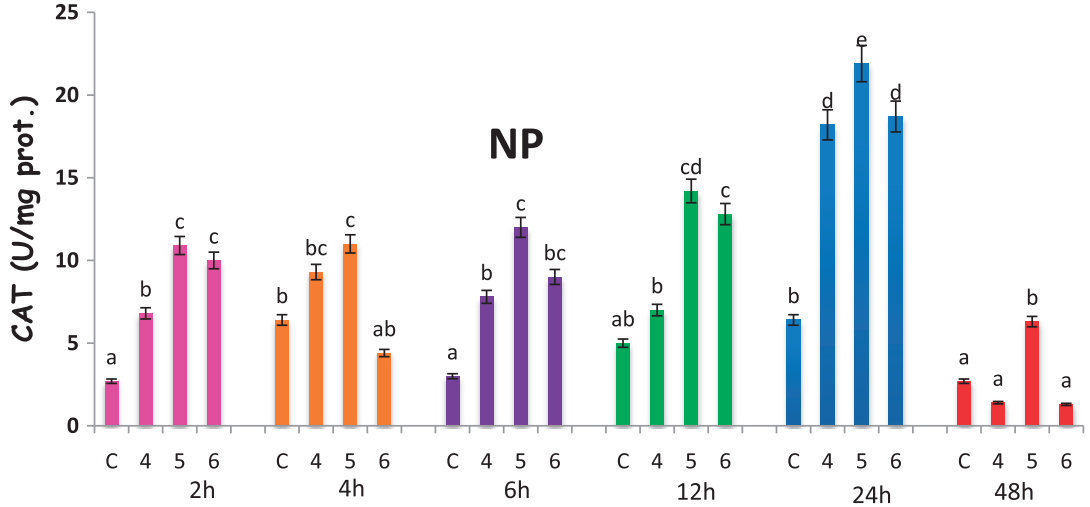



Figure 4. Time- and dose-dependent change in the CAT level in NP-applied hepatocyte culture (4, 5, 6: nonylphenol $10^{-4}$, $10^{-5}$, $10^{-6}$ M). The difference between the columns with different letters is significant ($p < 0.05$) (The statistics letters show the differences between the concentrations).

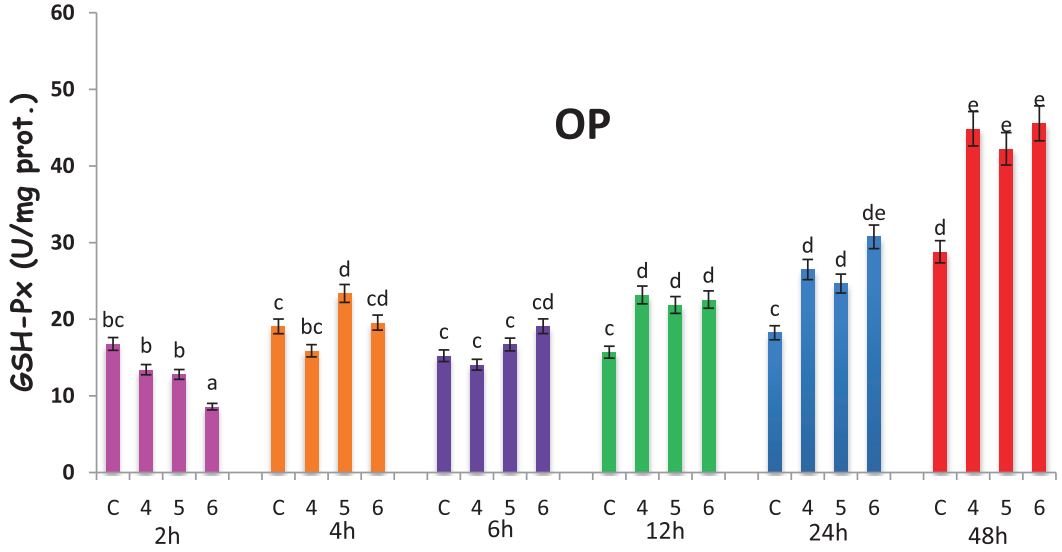



Figure 5. Time- and dose-dependent change in the GSH-Px level in OP-applied hepatocyte culture (4, 5, 6: octylphenol $10^{-4}$, $10^{-5}$, $10^{-6}$ M). The difference between the columns with different letters is significant ($p < 0.05$) (The statistics letters show the differences between the concentrations).

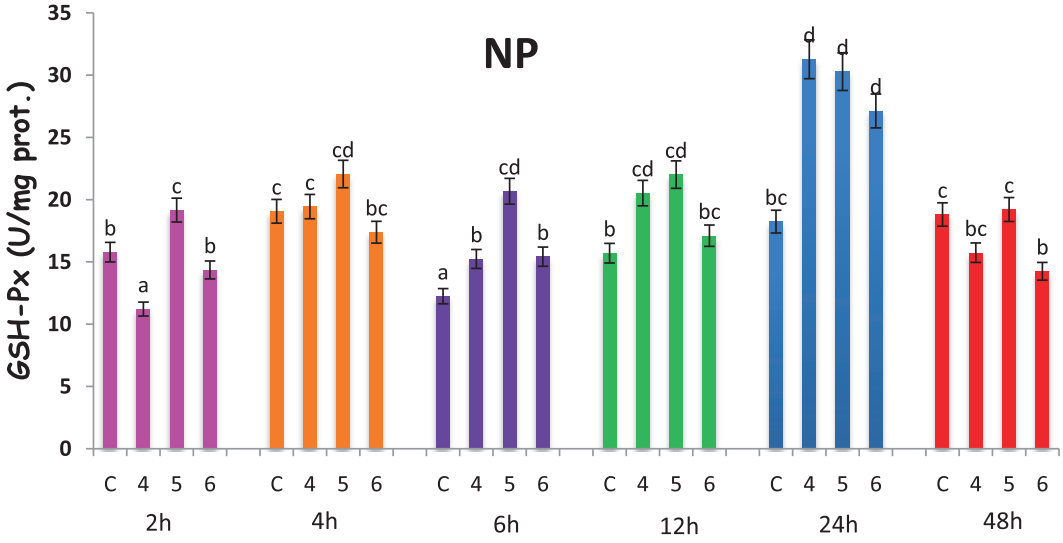



Figure 6. Time- and dose-dependent change in the GSH-Px level in NP-applied hepatocyte culture (4, 5, 6: nonylphenol $10^{-4}$, $10^{-5}$, $10^{-6}$ M). The difference between the columns with different letters is significant ($p < 0.05$) (The statistics letters show the differences between the concentrations).

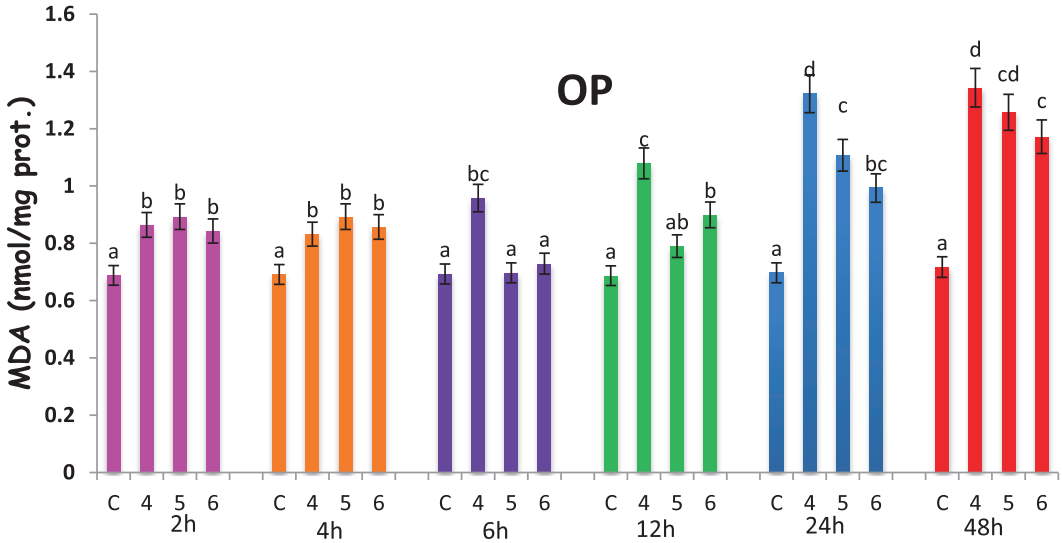



Figure 7. Time- and dose-dependent change in the MDA level in OP-applied hepatocyte culture (4, 5, 6: octylphenol $10^{-4}$, $10^{-5}$, $10^{-6}$ M). The difference between the columns with different letters is significant ($p < 0.05$) (The statistics letters show the differences between the concentrations).

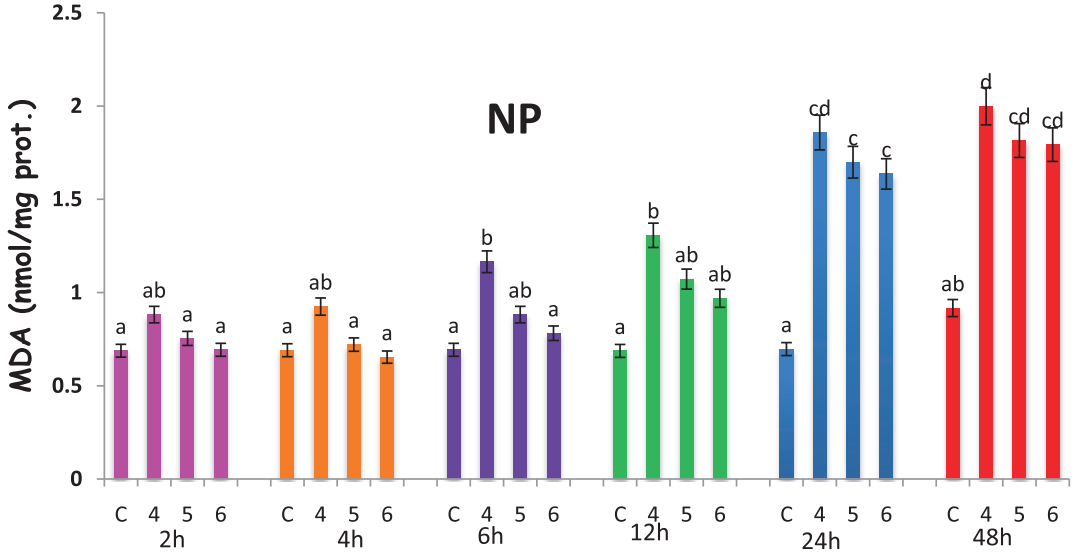



Figure 8. Time- and dose-dependent change in the MDA level in NP-applied hepatocyte culture (4, 5, 6: nonylphenol $10^{-4}$, $10^{-5}$, $10^{-6}$ M). The difference between the columns with different letters is significant ($p < 0.05$) (The statistics letters show the differences between the concentrations).

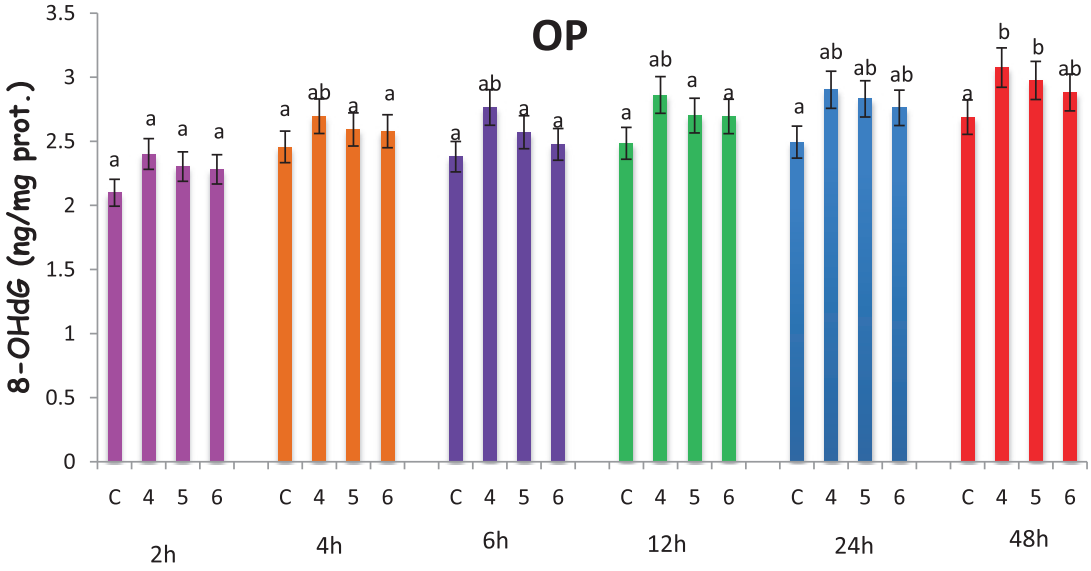



Figure 9. Time- and dose-dependent change in the 8-OHdG level in OP-applied hepatocyte culture (4, 5, 6: octylphenol $10^{-4}$, $10^{-5}$, $10^{-6}$ M). The difference between the columns with different letters is significant ($p < 0.05$) (The statistics letters show the differences between the concentrations).

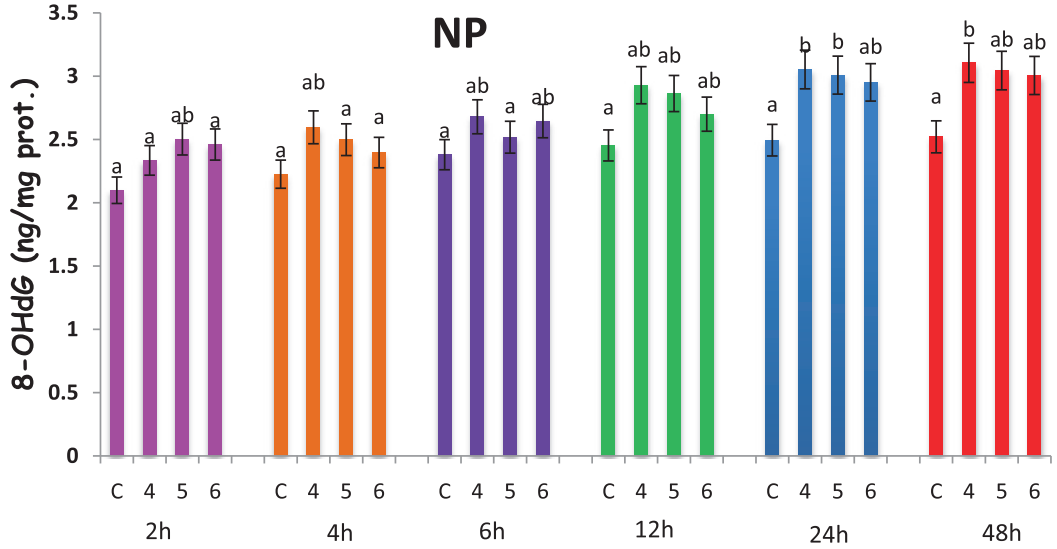



Figure 10. Time- and dose-dependent change in the 8-OHdG level in NP-applied hepatocyte culture (4, 5, 6: nonylphenol $10^{-4}$, $10^{-5}$, $10^{-6}$ M). The difference between the columns with different letters is significant ($p < 0.05$) (The statistics letters show the differences between the concentrations).

1968 ng/L, 605 ng/L, 522 ng/L, and 1105 ng/L in the Mira River, respectively (Rocha et al. 2016).

The effects of alkylphenol degradation products on living organisms in nature have been demonstrated in many studies (Folmar et al. 1996, Rudel et al. 1998). Exposure to relatively high concentrations of OP produced histopathological changes in the testes of *C. dimerus*, generating infertile males (Rey et al. 2016). In this study, OP and NP were demonstrated to cause oxidative stress in the primary hepatic cell culture of Van Fish. The SOD level was observed to increase during the first 24 hours and decrease at the 48th hour after exposure to both chemicals. 4-NP causes oxidative damage due to reactive oxygen species (ROS), and endocrine toxicity in catfish exhibits a detrimental effect showing the relationship between enzymatic and hormonal systems for defense (Sayed and Ismail 2017). In this study, activities of the examined SOD and CAT exhibited significant elevation after applying the NP treatment. Catfish exposed to NP serum and hepatic tissue produced similar results (Park 2015, Sayed and Hamed 2017). *Paralichthys olivaceus* bronchial cell culture was exposed to NP, and the change in antioxidant level was monitored. The SOD, CAT, and GSH-Px levels first increased, and then decreased (Xiao et al. 2007). In a similar study, oxidative stress was induced in the muscular tissue of Japan Sea bass via 17β estradiol administration. The SOD, CAT, GSH-Px, and MDA levels were observed to increase, while no significant change was observed in terms of DNA damage (Thilagam, et al. 2010). The outcomes of these studies are parallel to the data obtained in this study.

Increased DNA damage may also lead to apoptosis and apoptosis was observed in fish sterol cells when exposed to NP (Weber et al. 2002, Miura et al. 2005, Yi et al. 2009). It was demonstrated that some chemicals applied on the embryos of zebrafish increased the 8-OHdG levels 144 hours following the application (Zhu et al. 2016). In another study, the effect of NP on DNA damage may also depend on the activity of microtubule disruptors (Vazquez-Duhalt et al. 2005) or DNA levels that can cause changes in the reactive intermediates for their biotransformation (Sharma and Chadha 2017). At the end of 56 days, the 8-OHdG levels were observed to remain unchanged from the beginning to end; however, it decreased following lysine administration while the MDA level increased (Li et al. 2016). In a study of rats, lipid peroxidation was observed to increase in the time following OP administration, and antioxidant levels were observed to decrease (Morgan et al. 2014). 4-NP may cause DNA damage due to adduct formation, ROS formation, micro tubular disruption activity, and also by impairment of the DNA repair system (Sharma and Chadha 2017). In one study, the larvae of zebrafish were exposed to different doses of pentachlorophenol and $TiO_2$ for 6 days, and SOD, MDA, and DNA damage were detected. It was observed that the 8-OHdG level in the larvae was changed only at the highest dose and in the latest period (Fang et al. 2015). The outcomes of these studies are consistent with the data obtained in this study. Also, there was a significant increase in MDA after 4-NP exposure; this increase can be a result of the impairment of antioxidant enzymes due to the ROS formation that attacks the cell membrane. Furthermore, DNA damage being observed only at the end of the studies indicates that DNA damage may be observed as a result of long-term exposure to toxic agents only. These data suggest that DNA damage could have been observed in the long-term in this study as well.

The antioxidants (SOD, CAT, and GSH-Px) were generally affected by OP and NP application in this study. Furthermore, the antioxidants were observed to increase compared to the control group, starting at the beginning of the study, and changing according to the concentrations of the chemicals used. In particular, a significant increase was observed in the levels of SOD and CAT at the 24th hour. CAT levels were significantly increased in each group, especially at OP4 ($10^{-4}$ M) concentration (Figures 1–4). This indicates that hepatic metabolism induced an increase in the levels of SOD and CAT to provide antioxidants in response to the increased level of radicals. The levels of SOD and CAT were decreased in both OP and NP applications at the end of the 48th hour. The reduction in antioxidant defenses at 48 hours can be attributed to the depletion of living hepatocyte cells and chemically induced denaturation. The GSH-Px level, on the other hand, was observed to increase at the 24th and 48th hours following OP application, and increased at the 24th hour while it decreased at the 48th hour following NP application. This may have resulted from different levels of GSH-Px activity following NP and OP applications or from the reduction in cell survival at the 48th hour. In addition, GSH-Px activity decreased after both chemicals compared to the control group. This may be due to a partial inhibition of GSH-Px activity by the chemicals. The increase observed in the level of MDA following OP and NP applications at the 24th hour indicates an increased level of free radicals and lipid peroxidation. The increase in the levels of antioxidants at the 24th hour supports this suggestion as well. It was also observed that MDA levels at $10^{-4}$ M concentration showed a significant increase after both chemicals were applied. This indicates that the MDA level is affected considerably as the chemical concentration increases (Figures 7 and 8). The 8-OHDG level was slightly increased compared to the control group, but the increases were evident at 24 and 48 hours (Figures 9 and 10). As mentioned before, this suggests that DNA damage is observed after longer-term exposure to toxic substances. It may be concluded that after longer-term exposure at higher doses to agents that disrupt the endocrine system, the levels of antioxidants and MDA would be increased, and DNA damage would be observed. In this study, it was observed that the levels of OP induced high antioxidants and high NP chemical affected MDA and 8-OHDG levels. This may be due to an inhibitory effect of NP on antioxidant metabolism.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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