Chemosphere 204 (2018) 156–162

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Chronic toxicity and endocrine disruption of naproxen in freshwater waterfleas and fish, and steroidogenic alteration using H295R cell assay

Kyunghee Kwak [a], Kyunghee Ji [b, *, 2], Younglim Kho [c], Pilje Kim [d], Jaean Lee [d], Jisung Ryu [d, 1], Kyungho Choi [a, **, 2]

[a] School of Public Health & Institute of Health and Environment, Seoul National University, Seoul, 08826, South Korea
[b] Department of Occupational and Environmental Health, Yongin University, Yongin, 17092, South Korea
[c] Department of Health, Environment and Safety, Eulji University, Seongnam, Gyeonggi, 13135, South Korea
[d] National Institute of Environmental Research, Incheon, 22689, South Korea

HIGHLIGHTS

- Chronic exposure to naproxen impaired reproduction in crustaceans.
- Significant decrease of juvenile survival of medaka was observed at 0.5 mg L^{-1}.
- Naproxen altered gene transcription related to steroidogenesis in juvenile fish.
- Levels of E2 were significantly increased in H295R cells exposed to naproxen.
- Potential ecological risks by naproxen cannot be ruled out in some hotspots.

ARTICLE INFO

Article history:
Received 23 January 2018
Received in revised form
1 April 2018
Accepted 6 April 2018
Available online 7 April 2018

Handling Editor: Shane Snyder

Keywords:
Daphnia magna
Endocrine disruption
Naproxen
Oryzias latipes
Reproduction

ABSTRACT

Naproxen is a non-steroidal anti-inflammatory drug (NSAID) and has been frequently detected in surface waters around the world. Although endocrine disruption and reproduction related effects of NSAIDs are of increasing concern, the ecotoxicity of naproxen in aquatic organisms is limited primarily to acute lethal effects. In this study, chronic toxicity of naproxen was evaluated employing two daphnids (*Daphnia magna* and *Moina macrocopa*) and a fish (*Oryzias latipes*). The effects of naproxen on sex steroid hormones and gene transcription related to steroidogenesis were also evaluated in H295R cells. The chronic no observed effect concentrations (NOECs) of naproxen for reproduction were determined to be 10 mg L^{-1} in *D. magna* and 0.3 mg L^{-1} in *M. macrocopa*. At concentrations of 0.5 mg L^{-1}, the survival of juvenile medaka fish was significantly decreased and transcription of *erβ2* gene was significantly increased. Concentration of 17β-estradiol (E2) and the ratio of E2 and testosterone were significantly increased in H295R cells at 10 mg L^{-1}, suggesting that naproxen could modulate sex hormone production. The current detected levels of naproxen in ambient Korean rivers are far lower than the effective levels, however potential adverse effects cannot be ignored in some highly polluted areas. Endocrine disruption effects in fish warrant further investigation particularly for their ecological implications.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) have been widely used for their analgesic, antipyretic, and anti-inflammatory properties (Hayashi et al., 2008). The most popular NSAIDs include acetylsalicylic acid, ibuprofen, and naproxen, and these are available over the counter in most countries (Nishi et al., 2015). Due to its widespread use, naproxen has been detected frequently in both

* Corresponding author. Department of Occupational and Environmental Health, Yongin University, Yongin, 17092, South Korea.
** Corresponding author. School of Public Health & Institute of Health and Environment, Seoul National University, Gwanak, Seoul, 08826, South Korea.
E-mail addresses: kyungheeji@yongin.ac.kr (K. Ji), kyungho@snu.ac.kr (K. Choi).
[1] Current address: Accident Prevention and Assessment Division, National Institute of Chemical Safety, Daejeon, 34111, Republic of Korea.
[2] Dr. Ji and Choi contributed equally to this work as corresponding authors.

https://doi.org/10.1016/j.chemosphere.2018.04.035
0045-6535/

sewage treatment plant effluents and surface waters worldwide. For example, naproxen has been detected in surface waters at concentrations up to 32 μg L^{-1} in Pakistan, 4.5 μg L^{-1} in Canada, 0.328 μg L^{-1} in China, and 0.24 μg L^{-1} in Japan (Brun et al., 2006; Komori et al., 2013; Selke et al., 2010; Zhao et al., 2010). In Korean rivers, naproxen was detected up to 0.326 μg L^{-1} (National Institute of Environmental Research (NIER), 2011).

Despite its frequent detection in aquatic environments, ecotoxicological information on naproxen is focused mostly on acute toxicity, and their median effective or lethal concentrations (EC_{50}s or LC_{50}s) were generally in the range of 66.4–625.5 mg L^{-1}. For example, previous studies reported that EC_{50} in algae (*Scenedesmus quadricauda*) after 72 h exposure was 101.45 mg L^{-1} (Ding et al., 2017) and in *Daphnia magna* was 46.72 mg L^{-1} after 48 h exposure (Gheorghe et al., 2016). Acute toxicity data may be useful for accident spill areas, but may not reflect the real consequences of long-term exposure to pharmaceutical contamination. To date, limited information is available for chronic exposure effects of naproxen on aquatic organisms, except for algae (Cleuvers, 2004) and fish (Sehonova et al., 2017).

Concerns on endocrine disrupting toxicity of NSAIDs have been increasing. For example, exposure to mild analgesics (e.g., ibuprofen and acetylsalicylic acid) during fetal life is associated with anti-androgenic effects and "compensated hypogonadism" has been emphasized as one of the possible mechanisms (Kristensen et al., 2018). Previous studies have suggested that chronic exposure to naproxen could induce various adverse effects, including oxidative stress and endocrine disruption. For example, naproxen caused mild oxidative stress in adult zebrafish at concentrations of 0.001 mg L^{-1} (Stancová et al., 2015a), and moderate effects on the expression of antioxidant genes in the intestines (Stancová et al., 2015b). The ratio of 17β-estradiol (E2) to testosterone (T) was significantly elevated in zebrafish exposed to 0.01 mg L^{-1} for 21 d (Ji et al., 2013). Disruption of the endocrine system could cause changes in reproduction, however the information of endocrine disruption potential and underlying mechanisms of naproxen is very limited.

In this study, the chronic toxicity and endocrine disruption effects of naproxen were investigated using freshwater organisms. Two freshwater invertebrates (*D. magna* and *Moina macrocopa*) and a fish (*Oryzias latipes*) were used. *D. magna* is the most commonly used freshwater crustacean in toxicity tests and *M. macrocopa* is an indigenous water flea species in Korea (Oh and Choi, 2012). Japanese medaka (*O. latipes*) is ideal for studying long-term effects of endocrine disruption chemicals (Yamani, 2004). To investigate underlying mechanisms of endocrine disruption, we used the human adrenocortical carcinoma (H295R) cell line. The H295R assay has been validated by the U.S. Environmental Protection Agency for use in a tiered screening approach (Gracia et al., 2006). The results of this study can be used to understand potential risks of naproxen in aquatic ecosystems, and associated mechanisms of endocrine disruption.

2. Materials and methods

2.1. Test chemicals

Naproxen (CAS No. 22204-53-1) (Table S1) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were prepared in 0.1% DMSO and were used for toxicity evaluation after dilution with appropriate media for each test organism. The final concentration of DMSO in the test solution did not exceed 0.1% (v/v).

The actual concentrations in exposure medium were measured using high performance liquid chromatography tandem mass spectrometry (LC-MS/MS). Water samples were collected at the beginning of the exposure (new) and after the 48 h exposure (old) period in the highest concentration. Identification and quantification were performed by a triple quadrupole mass spectrometer with electrospray ionization in negative mode (API 4000 triple MS/MS system, Applied Biosystems, Forster City, CA, USA). Detailed information regarding analysis conditions and parameters are shown in Tables S2, S3, and Figure S1. The limit of detection of naproxen was 1.60 ng mL^{-1}.

2.2. Maintenance of test organisms

Crustaceans (*D. magna* and *M. macrocopa*) were cultured in M4 media in the Environmental Toxicology Laboratory at Seoul National University (Seoul, Korea). Japanese medaka (*O. latipes*) were cultured in filtered tap water after dechlorination by aeration for >24 h. Fish were maintained at 25 ± 1 °C under 16:8 h light/dark photoperiod and were fed twice a day with freshly hatched *Artemia nauplii* (Brine Shrimp Direct, Ogden, UT, USA). Water quality parameters, including dissolved oxygen, pH, conductivity, and temperature were monitored routinely. To confirm consistent sensitivity of the organisms over time, reference tests were conducted with zinc chloride monthly (data not shown).

2.3. Toxicity tests in D. magna and M. macrocopa

The 48 h acute toxicity tests with *D. magna* and *M. macrocopa* were performed following Organization for Economic Cooperation and Development (OECD) test guideline 202 (OECD, 2004). Immobilized organisms were recorded daily and median effective concentration (EC_{50}) was calculated after 48 h exposure.

The 21 d chronic toxicity test with *D. magna* and 7 d with *M. macrocopa* were conducted as outlined in OECD test guideline 211 (OECD, 2012) and Oh and Choi (2012), respectively. Based on the acute toxicity test results, exposure concentrations were determined. Control or treatment groups included ten replicates with one neonate each. Crustaceans were exposed to various concentrations of naproxen (0, 0.33, 1.1, 3.3, 10, or 30 mg L^{-1}). The media were changed every two days and water quality parameters were measured before and after renewal of the medium. Tested water fleas were fed once daily with *Chlorella*. The mortality of parent *Daphnia* and newborn neonates were recorded daily. At the end of the test, the body length of *D. magna* was measured using a stereomicroscope (Dongwon, Bucheon, Korea). The intrinsic rate of population growth (r) was calculated using the Euler-Lotka equation as described by Heugens et al. (2006). If parent *Daphnia* died during exposure or were determined to be male, then the replicate was excluded from the statistical analysis. The test protocol of *M. macrocopa* was similar to the *D. magna* chronic toxicity test except for the exposure temperature (25 ± 1 °C) and test duration (7 d).

2.4. Early life stage (ELS) tests in fish

Fish ELS toxicity tests using Japanese medaka (*O. latipes*) were performed following OECD test guideline 210 (Organization for Economic Cooperation and Development (OECD), 1992). Fertilized fish eggs (n = 60, divided equally in four replicate test chambers) were exposed to 0, 0.005, 0.05, 0.5, 5, and 50 mg L^{-1} concentrations of naproxen in 50 mL glass beakers. After hatching, newly hatched larvae were transferred to 250 mL glass beakers. Hatchability, time to hatch, and fish survival were recorded and dead fish were removed as soon as possible. Larvae and juvenile fish were fed with flake food (Topmeal®) or newly hatched *Artemia* nauplii *ad libitum* whilst minimizing the surplus. At 30 days post hatch (dph), length and dry weight of juvenile fish were measured.