Endocrine disruption and consequences of chronic exposure to ibuprofen in Japanese medaka (Oryzias latipes) and freshwater cladocerans Daphnia magna and Moina macrocopa

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**ABSTRACT**

Despite frequent detection of ibuprofen in aquatic environments, the hazards associated with long-term exposure to ibuprofen have seldom been investigated. Ibuprofen is suspected of influencing sex steroid hormones through steroidalgenic pathways in both vertebrates and invertebrates. In this study, the effect of ibuprofen on sex hormone balance and the associated mechanisms was investigated in vitro by use of H295R cells. We also conducted chronic toxicity tests using freshwater fish, Oryzias latipes, and two freshwater cladocerans, Daphnia magna and Moina macrocopa, for up to 144 and 21 d of exposure, respectively. Ibuprofen exposure increased 17β-estradiol (E2) production and aromatase activity in H295R cells. Testosterone (T) production decreased in a dose-dependent manner. For D. magna, the 48 h immobilization EC50 was 51.4 mg/L and the 21 d reproduction NOEC was <1.23 mg/L. For M. macrocopa, the 48 h immobilization EC50 was 72.6 mg/L and the 7 d reproduction NOEC was 25 mg/L. For O. latipes, 120 d survival NOEC was 0.0001 mg/L. In addition, ibuprofen affected several endpoints related to reproduction of the fish, including induction of vitellogenin in male fish, fewer broods per pair, and more eggs per brood. Parental exposure to as low as 0.0001 mg/L ibuprofen delayed hatching of eggs even when they were transferred to and cultured in clean water. Delayed hatching is environmentally relevant because this may increase the risk of being predated. For O. latipes, the acute-to-chronic ratio of ibuprofen was estimated to be greater than 1000. Overall, relatively high acute-to-chronic ratio and observation of reproduction damage in medaka fish at environmentally relevant ranges of ibuprofen warrant the need for further studies to elucidate potential ecological consequences of ibuprofen contamination in the aquatic environment.

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**1. Introduction**

Pharmaceuticals are developed and used for intended biological effects in human and veterinary medicine. The physiologically active nature of pharmaceuticals, however, raised concerns about their potential impacts to non-target species when they were inadvertently discharged into ecosystem (Ankley et al., 2007; Fent et al., 2006). Ibuprofen (RS)-2-(4-isobutylphenyl)propanoic acid, CAS number 15687-27-1) is one of non-steroidal anti-inflammatory drugs (NSAIDs), and is widely used as analgesic, anti-pyretic and anti-inflammatory purposes to relieve symptoms of arthritis, rheumatic disorders and fever (Hayashi et al., 2008). It is one of the

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Abbreviations: cAMP, cyclic adenosine monophosphate; CF, condition factor; COX, cyclooxygenase; CI, confidence interval; CYP, cytochrome P450; dph, day post-hatch; EC50, median effective concentration; ELISA, enzyme-linked immunosorbent assay; GSI, gonadosomatic index; HSI, hepatosomatic index; LOEC, lowest observed effect concentration; MCIG, minimum concentration to inhibit growth; NOEC, no observed effect concentration; NSAID, non-steroidal anti-inflammatory drug; PCE2, prostaglandin E2; PGFR, population growth rate; STP, sewage treatment plant.

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core medicines included in “Essential Drugs List” of World Health Organization, and therefore produced in large amounts worldwide (Heckmann et al., 2007).

Ibuprofen has frequently been detected in surface water, with as much as 0.1 μg/L detected in surface water of South Wales, UK (Kasprzyk-Hordern et al., 2008). The average concentration detected in major rivers of Korea was 0.03 μg/L (Kim et al., 2007). Relatively greater concentrations of ibuprofen have been reported for effluents (up to 22 μg/L) and influents (up to 84 μg/L) of sewage treatment plants (STPs) (Brun et al., 2006; Gómez et al., 2007). Hence municipal wastewater effluents are an important source of ibuprofen in aquatic environments, especially streams and rivers.

Due to the widespread occurrence of ibuprofen in aqueous environments, its potential for ecological impact has been of growing concern (Christensen et al., 2009). Ibuprofen is known to influence the reproductive-related endpoints such as blood size and number of cells, human adrenocortical carcinoma cell line and three model freshwater species, including freshwater macroinvertebrates D. magna and M. macrocopa, and fish O. latipes, were employed.

2. Material and methods

2.1. Chemicals

Test solutions were freshly prepared by diluting stock solutions with appropriate culture media, immediately before the test or before the renewal of test solutions. Ibuprofen (purity 98%) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Solvent-free stock solution of ibuprofen was prepared by dissolving in MilliQ water (Millipore Asia, Yonezawa, Japan) with sonication. Measured concentrations of ibuprofen were 1.13 and 71.9 mg/L for the nominal concentrations of 1 and 100 mg/L, respectively. Concentrations of ibuprofen did not change more than 6% after the 48 h of exposure of D. magna or O. latipes (Supplement Table S1). Nominal concentrations are reported throughout the paper.

2.2. Cell culture and maintenance of test organisms

The H295R human adrenocortical carcinoma cell line was obtained from the American Type Culture Collection (ATCC # CRL-2128, ATCC, Manassas, VA, USA) and cultured at 37 °C in a 5% CO2 atmosphere as previously described (Gracia et al., 2007). Briefly, the cells were grown in a 1:1 mixture of Dulbecco’s Modified Eagle’s Medium and Ham’s F-12 Nutrient mixture (DMEM/F12) (Sigma D-2906; Sigma–Aldrich) supplemented with 1.2 g/L Na2CO3, 10 mL/L of ITS+ Premix (BD Bioscience; 354352), and 25 mL/L of Nu-Serum (BD Bioscience; 355100). Medium was changed every 4 d and cells were subcultured every week.

The two freshwater cladocerans (D. magna and M. macrocopa) and the fish (Japanese medaka fish; O. latipes) were cultured and maintained in the Environmental Toxicology Laboratory, Seoul National University (Seoul, Korea) following US Environmental Protection Agency (US EPA, 2002), Oh (2007), and Organization for Economic Cooperation and Development TG 203 (OECD, 1992a,b) protocols, respectively. Both crustaceans were fed daily with a 1:1:1 mixture of yeast (ACH Food Companies, Memphis, TN, USA), cerophyll (Nutraceutical Corporation, Park City, UT, USA), and Tetramin® (Tetra, Melle, Germany). In addition, algae (Pseudokirchneriella subcapitata) were also provided. Japanese medaka fish were cultured in filtered tap water after dechlorination for aeration for more than 24 h. Medaka were maintained at 25 ± 1 °C, under a 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 and logged whenever new batches of media were prepared, following American Public Health Association, American Water Works Association, and Water Pollution Control Federation (1992) protocols. To confirm comparable sensitivity of the test organisms over time, acute lethality tests were conducted with the reference toxicant (zinc chloride) to determine the relative sensitivity of D. magna, M. macrocopa, and O. latipes on a monthly basis (data not shown).

2.3. Steroidogenesis assay using H295R cells

H295R cells were seeded into 24-well plates at a concentration of 3 × 10^4 cells/mL in 1 mL of medium per well. After 24 h, cells were exposed to ibuprofen dissolved in dimethyl sulfoxide (DMSO; Sigma–Aldrich, St. Louis, MO, USA). The final DMSO concentration in the exposure medium was less than 0.1% (v/v). H295R cells were exposed to various concentrations of ibuprofen for 48 h, and were inspected microscopically for viability. When the exposure resulted in cell viability less than 85%, the data were not used to determine the effect on hormone production (Gracia et al., 2007). In addition, to identify the range of ibuprofen concentrations that are non-cytotoxic, a Live/Dead cell viability assay kit (Molecular Probes, Eugene, OR, USA) was used. The culture medium was collected after the exposure and stored at −80 °C for further measurement of hormones produced.

Hormone extraction and quantification were conducted as previously described (Hecker et al., 2006). Briefly, hormones were extracted from the culture medium twice with diethyl ether (5 mL) and the solvent was evaporated under a gentle stream of nitrogen. The residue was reconstituted in ELISA assay buffer and was measured by competitive ELISA following the manufacturer’s recommendation (Cayman Chemical, Ann Arbor, MI, USA; Testosterone [T, Cat # 582701], 17β-Estradiol [E2, Cat # 582251]). Activity of aromatase enzyme was measured using two different methods, direct or indirect assays as previously described.
Fig. 1. Experimental procedures for chronic O. latipes toxicity test. The 12th day after egg exposure is assigned as the first day post-hatch (dph). Bold letter expresses the development stage. Number of fish that were observed for each endpoint is indicated above the arrows. At 90 dph, four mating pairs from the control or each treatment were selected for the examination of effects on reproduction. To investigate effects of ibuprofen on progeny generation (F1), eggs from four mating pairs were collected and transferred into clean culture water, and fertility, hatchability and time to hatch were determined. (Sanderson et al., 2001; Higley et al., 2010). For the experiments measuring direct effects of chemicals on aromatase activity, cells were exposed to various concentrations of ibuprofen in a medium containing 54 nM 1β-3[H]-androstenedione (Perkin Elmer, Boston, MA, USA), with no pre-exposure to ibuprofen. In order to measure indirect effects of ibuprofen on aromatase activity, H295R cells were pre-exposed to ibuprofen for 48 h, and then cells were washed twice and incubated with 0.25 mL of supplemented medium containing 54 nM 1β-3[H]-androstenedione. DMSO was used as carrier solvent and did not exceed 0.1% (v/v). After 1.5 h incubation at 37 °C with 5% CO2, cells were placed on ice to stop the reaction. A 200 μL aliquot of the cell suspension was removed, and chloroform and dextran-coated charcoal were added to remove all remaining 1β-3[H]-androstenedione. Aromatase activity was determined by the rate of conversion of 1β-3[H]-androstenedione to estrone. The quantity of 3[H] in extracts of medium was determined by LS 6500 multipurpose scintillation counter (Beckman Coulter, Fullerton, CA, USA). Aromatase activity was expressed as pmoles of androstenedione converted per h per 100,000 cells. Forskolin (10, 1, or 0.1 μM) was used as a positive control for aromatase induction, while prochloraz (10, 1, or 0.1 μM) was used as a negative control for aromatase catalytic inhibition.

2.4. D. magna and M. macrocopa toxicity tests

The 48 h immobility tests were conducted with D. magna and M. macrocopa to determine the range of ibuprofen concentrations for chronic survival and reproduction tests. Procedures used for 48 h immobility test were in accordance with US EPA (2002). The endpoints for the chronic tests were survival of original neonates, number of young per female, the number of young per brood, time to first reproduction, and population growth rate (PGR). PGR was calculated following the method used in Park and Choi (2008). Basic water chemistries such as dissolved oxygen, pH, temperature, and conductivity were measured and recorded before and after the medium renewal. Chronic D. magna toxicity tests (21 d) were conducted as outlined in OECD TG 211 (OECD, 2008). Ten replicates with one neonate each (<24 h old) were exposed to various concentrations of ibuprofen (0, 1.23, 3.70, 11.1, 33.3, or 100 mg/L). Test solutions were renewed three times per week. D. magna were fed daily with 300 μL YCT and 300 μL algae per each organism. Chronic M. macrocopa toxicity tests (7–8 d, three brood) were performed following the protocol by Oh (2007). The test method was similar to the chronic D. magna test method except for the test duration (21 d versus 7–8 d) and temperature (20 ± 1°C). Ten
Fig. 2. Mean concentrations of (a) estradiol (E2) and (b) testosterone (T) in H295R cell medium after 48 h exposure to ibuprofen. Error bar represents 1 standard deviation. Asterisk denotes a significant difference from the control (*p < 0.05; **p < 0.01). The p value shown on the figure indicates significance of trend line: Significant positive trend was observed for estradiol (p = 0.003), while a negative trend was observed for testosterone (p = 0.022).

replicate test chambers each containing a single neonate (<24 h old) were exposed to various concentrations of ibuprofen (0, 3.13, 6.25, 12.5, 25.0, or 50.0 mg/L).

2.5. O. latipes life cycle toxicity test

A full life cycle chronic toxicity test with a supplementary reproduction test was conducted with fertilized eggs (<24 h of spawning) for 144 d (Fig. 1). This test was an enhancement of the OECD embryo-larval test (TG 210), and was designed to measure gross development, vitellogenin induction, histological manifestations, and reproduction success following the chronic exposure to ibuprofen. During the exposure, fishes were fed freshly hatched A. nauplii twice a day.

Initially, 60 fertilized eggs were randomly separated into four test beakers (i.e., 15 eggs per beaker) in each treatment. Fertilized eggs were exposed to 0.01, 0.1, 1, 10, 100, or 1000 μg/L ibuprofen for 12 d. When the eggs hatched, hatchlings were moved into 250 mL beakers. Survival of the juvenile fish was monitored daily until 30 d post-hatch (dph). After 90 and 120 dph, effects on survival, condition factor, histopathology, or vitellogenin induction were determined. To evaluate reproduction effects, four pairs of fish were chosen from each treatment or control after 90 dph, and reproduction endpoints of each mating pair were determined daily for 30 d during the period of 90–132 dph. After this observation, all surviving pairs were euthanized and measured for the condition factor (CF), gonadosomatic index (GSI), and hepatosomatic index (HSI). In addition, the eggs that had been collected from the mating pairs were transferred to clean culture water and were observed for fertility, hatchability and time-to-hatch for additional 20 d.

Vitellogenin in blood plasma was measured using a kit (EnBio Medaka Vitellogenin ELISA system, COSMO BIO, Tokyo, Japan) according to the manufacturer’s instructions. A microtiter plate spectrophotometer (Tecan SPECTRAFluor, Tecan, Männedorf, Switzerland) was employed for the measurement. For histological observation, fish were euthanized and fixed in Bouin’s solution for 24 h, dehydrated in a series of ethanol and xylene baths, and embedded in paraffin. H&E stained tissue sections of gonads, livers, and kidneys were examined for histopathology under a light microscope.

2.6. Statistical analysis

The median effective concentrations (EC50) and associated 95% confidence intervals (CI) were calculated by Probit analysis or Spearman–Karber analysis depending on the normality of distribution and homogeneity of variance of the data. No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) were calculated using Fisher’s Exact test or Dunnett’s one-way analysis of variance (ANOVA) test. For analysis of reproduction data, outliers were identified using Dixon’s Q-test (Dean and Dixon, 1951). SPSS 12.0K for Windows (SPSS, Chicago, IL, USA) and ToxStat (ver 3.5. West, Cheyenne, WY, USA) were used.

3. Results

3.1. Hormone production and aromatase activity in H295R cells

Ibuprofen significantly increased the production of E2 in H295R cells in a dose-dependent manner (p = 0.003, Fig. 2a). Compared to
control, significantly greater E2 production was observed at concentrations of ibuprofen as little as 2 mg/L and E2 production as proportional to ibuprofen concentration up to 20 mg/L. T concentrations were inversely proportional to ibuprofen concentration up to 20 mg/L. T concentrations of ibuprofen as little as 2 mg/L and E2 production as significantly greater at 10 and 100 mg/L (Table 3). The number of eggs per brood exhibited a dose-dependent negative relationship.

3.2. Effects in Daphnia and fish

Ibuprofen cause statistically significant effects on survival and reproduction of both medaka and two freshwater cladocerans. The 48 h EC50s were estimated to be 51.4 mg/L (95% confidence interval 40.2–62.7) and 72.6 mg/L (55.0–90.4) for D. magna and M. macrocopa, respectively. Chronic effects of ibuprofen on survival and reproduction of D. magna and M. macrocopa exposure are summarized in Table 1. After 21 d exposure with D. magna, the NOEC based on survival of the initial neonate was 33.3 mg/L, while the LOEC based on reproduction was 1.23 mg/L. Significant populations decrease was observed in A. italicus when exposed to 100 mg/L (p < 0.01), but there was no dose-dependent relationship (Fig. 5). Also, because such a change was not observed among male fish, the response was most likely spurious. Histopathological observations of liver, gonad, and kidney found no lesions in exposed fish (Supplement Fig. S1).

Reproduction of eggs was not affected by either of the ibuprofen concentrations. However, the number of broods per pair exhibited a dose-dependent negative relationship.

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![Fig. 4. Blood VTG concentrations in blood plasma of male Oryzias latipes among ibuprofen treatments for 120 dph. Error bars represent standard deviation of level of vitellogenin. Vitellogenin level was expressed by logarithmic scale. Asterisk indicates a notable difference from the control (p < 0.1) based on Dunnett’s ANOVA test. Outliers were controlled by Q test (Dean and Dixon, 1951).](image-url)
and GSI are calculated as: gonad weight/total body weight × 100. Effect of ibuprofen exposure on hatchability and survival of fry, juvenile and adult medaka, b, c. Table 2. Gonadosomatic Index (GSI) and hepatosomatic Index (HSI) of male (a) and female (b) Japanese medaka exposed to various concentrations of ibuprofen for 132 dph. Fig. 5. Gonadosomatic Index (GSI) and hepatosomatic Index (HSI) of male (a) and female (b) Japanese medaka exposed to various concentrations of ibuprofen for 132 dph. Table 3. Effect of ibuprofen exposure on reproduction profile of medaka, b, c. Table 4. Fertility, hatchability, and time-to-hatch of progeny generation (F1) observed for 20 d in control water, a, b, c.
ship with exposure concentration ($p$ for trend < 0.05). Hatching was delayed among the eggs that were spawned from pairs which had been exposed to 1 g/L or greater. However, fertility and hatchability of the eggs were not affected by exposure to ibuprofen (Table 4).

**4. Discussion**

**4.1. Hormone production and aromatase activity in H295R cells**

Exposure of H295R cells to ibuprofen leads to not only greater production of E2 but also an increase in aromatase activity in a concentration-dependent manner (Figs. 2 and 3). Increased aromatase activity could facilitate the conversion of T to E2 and therefore lead to a reduction of T. In fact, concentrations of T were inversely proportional to concentration of ibuprofen to which cells were exposed (Fig. 2b). Ibuprofen did not directly affect the activity of aromatase (Brueggemeier et al., 2005). Aromatase (CYP19) is a cytochrome P450 enzyme that converts androstenedione into estrone (E1), or T into E2. Because E1 and E2 are two major estrogens in humans, inhibition of aromatase activity could reduce the concentration of estrogens and influence hormone balance and the physiological processes and behaviors that they control.

The mechanisms of E2 increase by ibuprofen exposure are not clear. One possibility is that pathways other than the COX enzyme might be associated with the ibuprofen-caused increase in aromatase activity and subsequent production of E2 by H295R cells. Another possibility is an interspecies variation of COX enzyme activity in response to ibuprofen exposure. Although the COX enzyme is known to influence synthesis of eicosanoids in both vertebrates and invertebrates (Hayashi et al., 2008), the effect of ibuprofen on COX activity does not appear to be the same among organisms: Ibuprofen lead to reduction of COX activity in female medaka that were exposed at 100 $\mu$g/L for 6 weeks (Flippin et al., 2007). In D. magna, however, microarray experiments showed increased transcription of the COX gene after the exposure to ibuprofen (Brueggemeier et al., 2005). Ibuprofen is thought to inhibit the COX pathway via competition with its substrate, arachidonic acid for the active sites of COX. Inhibition of the COX pathway reduces the synthesis of important eicosanoids such as prostaglandin E2 (PGE2). PGE2 increases intracellular cyclic adenosine monophosphate (cAMP) levels which up-regulates aromatase expression, which in turn, increases conversion of T into E2, and reduced PGE2 synthesis results in inhibition of estrogen production through modulation of aromatase (Brueggemeier et al., 2005). Aromatase (CYP19) is a cytochrome P450 enzyme that converts androstenedione into estrone (E1), or T into E2. Because E1 and E2 are two major estrogens in humans, inhibition of aromatase activity could reduce the concentration of estrogens and influence hormone balance and the physiological processes and behaviors that they control.

It should be noted that the observed increase of E2 in plasma after the exposure to ibuprofen is contrary to what several other reports suggest. Ibuprofen and other NSAIDs can influence estrogen biosynthesis in different ways (Brueggemeier et al., 2005; Terry et al., 2004). Ibuprofen is thought to inhibit the COX pathway via competition with its substrate, arachidonic acid for the active sites of COX. Inhibition of the COX pathway reduces the synthesis of important eicosanoids such as prostaglandin E2 (PGE2). PGE2 increases intracellular cyclic adenosine monophosphate (cAMP) levels which up-regulates aromatase expression, which in turn, increases conversion of T into E2, and reduced PGE2 synthesis results in inhibition of estrogen production through modulation of aromatase (Brueggemeier et al., 2005). Aromatase (CYP19) is a cytochrome P450 enzyme that converts androstenedione into estrone (E1), or T into E2. Because E1 and E2 are two major estrogens in humans, inhibition of aromatase activity could reduce the concentration of estrogens and influence hormone balance and the physiological processes and behaviors that they control.

**Table 5**

Acute and chronic toxicity of ibuprofen to aquatic organisms.

<table>
<thead>
<tr>
<th>Test type</th>
<th>Species</th>
<th>Duration/endpoint</th>
<th>Conc. (CI) (mg/L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Scenedesmus subspicatus</td>
<td>72 h/Growth EC50</td>
<td>342.2 (242.4–471.5)</td>
<td>Cleuvers (2004)</td>
</tr>
<tr>
<td></td>
<td>Vibrio fischeri</td>
<td>15 minutes luminescence EC50</td>
<td>18.1</td>
<td>Farré et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>Lemma gibba</td>
<td>7d/Wet weight Frond number EC50</td>
<td>&gt;1</td>
<td>Brain et al. (2004)</td>
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<td></td>
<td>Lemma minor</td>
<td>7d/Growth EC50</td>
<td>4</td>
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<tr>
<td></td>
<td>Hydra vulgaris</td>
<td>7d/Survival NOEC</td>
<td>&gt;10</td>
<td>Pascoe et al. (2003)</td>
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<td></td>
<td>Hydra attenuata</td>
<td>96h/EC50</td>
<td>22.36</td>
<td>Quinn et al. (2008)</td>
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<tr>
<td></td>
<td>Planeris carinatus</td>
<td>72h/EC50</td>
<td>17.08 (5.9–72.3)</td>
<td>Pounds et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Daphnia magna</td>
<td>48h/Immobilization EC50</td>
<td>55.6</td>
<td>Knoll/BASF (2009)</td>
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<tr>
<td></td>
<td>D. magna</td>
<td>48h/Immobilization EC50</td>
<td>101.2 (89.2–114.9)</td>
<td>Cleuvers (2004)</td>
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<td>48h/EC50</td>
<td>132.6</td>
<td>Han et al. (2006)</td>
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<td></td>
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<td>51.44 (40.7–62.71)</td>
<td>This study</td>
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<td></td>
<td>Moina macrocopa</td>
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<td>This study</td>
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<td>Xenopus laevis</td>
<td>96h/Growth Deformity NOEC</td>
<td>20</td>
<td>Richards and Cole (2006)</td>
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<tr>
<td></td>
<td>X. laevis</td>
<td>96h/EC50 (MCIG)</td>
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<td>Richards and Cole (2006)</td>
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<td>Richards and Cole (2006)</td>
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<td>96h/Malformation (Thoracic Edema) EC50</td>
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<td>Chronic</td>
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ibuprofen (Heckmann et al., 2008). This suggests that ibuprofen might influence the COX pathway differently in medaka (fish) than in Daphnia (crustacean). Further investigations are needed for verifying sex hormone induction in medaka, and elucidating the underlying mechanisms of steroidogenic alteration of ibuprofen exposure.

4.2. Effects on Daphnia and fish

Longer durations of exposure (21 d) of D. magna to ibuprofen resulted in greater effects. The LOEC for effects on reproduction was 1.23 mg/L, which was the least concentration studied, while the 48 h immobility EC50 was 51.4 mg/L. However for M. macrocopa, reproduction NOEC was observed at 25 mg/L, and it was not much different from 48 h immobility EC50 of 72.6 mg/L. Acute and chronic effects of ibuprofen or reproduction of other aquatic invertebrates have been reported to be in the mg/L range (Table 5). Considering reproduction-related effects due to chronic exposure, the sensitivity of D. magna observed in this study was close to that of the freshwater gastropod Planorbis carinatus. However, M. macrocopa appeared to be more tolerant of chronic ibuprofen exposure compared to other invertebrates. Lethality of Daphnia would not be expected at concentrations of ibuprofen observed in the environment.


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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.aquatox.2010.02.013.

References