Comparative Biochemistry and Physiology, Part C 204 (2018) 63–70

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Two common mild analgesics have no effect on general endocrine mediated endpoints in zebrafish (*Danio rerio*)

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ARTICLE INFO

Keywords:
Aspirin
FSDT
NSAID
Paracetamol
Prostaglandin
Sex ratio
Vitellogenin

ABSTRACT

Mild analgesics such as acetylsalicylic acid (ASA) and acetaminophen (APAP) exert their pain-relieving effect in humans by inhibition of prostaglandin synthesis. Prostaglandins play key roles in developmental and reproductive processes in vertebrates, and in recent years, it has been suggested that weak analgesics might also act as endocrine disrupters. In a set of experiments we investigated if ASA and APAP affect well-established endocrine endpoints in zebrafish (*Danio rerio*), which is a commonly used model organism in the investigation of endocrine disrupting chemicals. Zebrafish were exposed to APAP (0.22, 2.3, and 30 mg L^{-1}) or ASA (0.2, 0.5, 1.7, and 8.2 mg L^{-1}) from hatch to sexual maturity in a test design resembling the OECD Fish Sexual Development Test. No effects on sex ratio and vitellogenin levels were observed. Adult zebrafish were exposed to high concentrations (mg L^{-1}) of ASA or APAP for eight or 14 days. ASA reduced the levels of prostaglandin E_2, but had no effect on the concentration of 11-ketotestosterone and vitellogenin.

Overall, ASA decrease prostaglandin E_2 concentrations, but well-established endpoints for endocrine disruption in zebrafish are generally not affected by aquatic exposure neither during development nor adulthood. According to the WHO/IPCS definition of an endocrine disrupter, the present results do not define APAP and ASA as endocrine disrupters.

1. Introduction

Mild analgesics are sold over-the-counter under various trade names, and the most common active ingredients in non-prescription mild analgesics are acetylsalicylic acid (ASA), ibuprofen, and paracetamol also called acetaminophen (APAP). The sales of mild analgesics have been increasing in many countries during the latest decades (Hudec et al., 2012; Kristensen et al., 2016). For example, the annual sale in Denmark in 2015, including both over-the-counter and by prescription sales, was 155,000,000 DDD (defined daily dose) corresponding to > 27 DDD per person in 2015 (The Danish Health Data Authority, 2017).

Mild analgesics exert their analgesics and antipyretic effects by inhibiting the synthesis of prostaglandins, which are modified fatty acids. Arachidonic acid is the primary precursor of prostaglandins, and it is converted into various prostaglandins by action of the bi-functional cyclooxygenase enzymes. There are two isoforms of the cyclooxygenase enzymes, Cox-1 and Cox-2, in humans and also in zebrafish (*Danio rerio*) (Grosser et al., 2002; Ishikawa et al., 2007). Prostaglandins play an important role in several reproductive processes such as ovulation and sexual behaviour in both mammals and fish (reviewed by Cha et al. (2006) and Corcoran et al. (2010)). Because prostaglandins play a role in the masculinisation of the male foetus during mammalian development concern has also been raised over human prenatal exposure to analgesics (reviewed by Kristensen et al. (2016)). Recently, mild analgesics were shown to have an endocrine disrupting potential during foetal life: intake of analgesics during pregnancy was associated with cryptorchidism in humans (Jensen et al., 2010; Kristensen et al., 2011), exposure to APAP during foetal development de-masculinised male rats (Kristensen et al., 2011; Hay-Schmidt et al., 2017), and anti-estrogenic effects were observed in testes of humans and rats exposed to mild analgesics (Jensen et al., 2010; Kristensen et al., 2011; Albert et al., 2013). Prostaglandins are involved in both ovulation and gonadal differentiation in zebrafish, and they have also been demonstrated to influence steroidogenesis in cultured goldfish testis (Wade and Van Der Kraak, 1993; Lister and Van Der Kraak, 2008; Pradhan and Olsson, 2014). Sexual development of fish is under strong influence of sex steroids, and several endocrine disrupters affect the sex ratio and yolk protein (vitellogenin) synthesis in zebrafish (Kinnberg et al., 2007; Morthorst et al., 2010; Holbech et al., 2012; Holbech et al., 2013), which are both well-established and recognised endocrine mediated endpoints included in the OECD Test Guideline 234 Fish Sexual

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https://doi.org/10.1016/j.cbpc.2017.11.009
Received 27 July 2017; Received in revised form 22 November 2017; Accepted 23 November 2017
Available online 26 November 2017
1532-0456/

Table 1
Overview of the FSDT and adult exposure experiments with APAP and ASA. dpf: days post fertilization; dph: days post hatch (3 dpf = 0 dph). Only 28% of the fish survived in the APAP adult exposure and further analyses were therefore not performed.
§ one of the replicates (3.8 mg L^{-1}) had 7 females and 9 males, because one male was mistaken as a female when sexing the fish before the experiment started.

Adult exposures	Duration	Replicates	Fish per tank (n)		Endpoints
ASA	8 days	1 replicate	10	Males only	Vtg
Control					
0.4 mg L^{-1}					
0.8					
3.0					
9.5					
ASA	14 days	2 replicates	16	8 females 8 males§	11-KT and PGE_2 in males
Control					
3.8 mg L^{-1}					
APAP	14 days	2 replicates	16	8 females 8 males	
Control					
1.9 mg L^{-1}	Most died				

FSDT exposures	Duration	Replicates	Fish per tank (n)	Sex ratio %(F:M)	Endpoints
ASA	3 dpf to 60 dph	3 replicates	80		Sex ratio and Vtg
Control				18:82	
0.2 mg L^{-1}				29:71	
0.5				29:71	
1.7				29:71	
8.2				30:70	
APAP	2 dpf to 74–75 dph	3 replicates	40		Sex ratio and Vtg
Control				21:79	
0.22 mg L^{-1}				34:64	
2.26				24:73	
30	(Died)				

Development Test (FSDT) (OECD, 2011). Because prostaglandins are involved in reproductive processes, gonadal differentiation, and steroid synthesis the prostaglandin inhibitors, such as APAP and ASA, might also affect sexual development of zebrafish, and therefore the aims of the experiments were to investigate 1) if exposure to APAP and ASA affected prostaglandin synthesis, steroid production and vitellogenin levels in adult zebrafish, and 2) if exposure to APAP and ASA during sex determination and differentiation affected sex ratio and vitellogenin levels.

Here we attempt to summarise the data obtained from of a series of experiments carried out over a period of several years during the mid 2000s, when the OECD test guideline 234 was still undergoing validation and information about e.g. the aquatic toxicity of analgesics was scarce.

2. Materials and methods

An overview of the experiments, including experimental details and endpoints, is provided in Table 1.

2.1. Chemicals

Acetaminophen (APAP) (99%, Cas No. 103-90-2) and acetyl salicylic acid (ASA) (99%, Cas No. 50-78-2) were purchased from Sigma-Aldrich (Vallensbæk Strand, Denmark). Stock solutions were made in ASTM type-1a water (Elga, PURELAB), and fresh stock solutions were prepared once or twice per week.

2.2. Animals and housing

Adult zebrafish were obtained from a local supplier (credofish.dk) and the fish were acclimatized for several weeks in the zebrafish unit at University of Southern Denmark in tap water (ground water) mixed with deionized water. The test systems in all experiments consisted of 8 L glass aquaria with 6 L of water and a water exchange of 18 L per 24 h (flow-through), and the test systems had been running for two days before the embryos or adult fish were added. The water temperature and oxygen saturation was measured once or twice per week. The temperature was 27 ± 1 °C and the average oxygen saturation level in the tanks was between 66 and 73% of the air saturation value (on the day of sampling single oxygen saturation levels of 55% were measured in the APAP FSDT because the pumps were paused for a few hours). The photoperiod was 14:10 h (light:dark), except in the ASA FSDT and the 8 days adult exposure where it was 12:12 h. The pH and conductivity of the water were kept within the recommended range for zebrafish husbandry (Brand et al., 2002). The supply of both water and test compounds was controlled by peristaltic pumps (Ole Dich Instrument Makers, Denmark).

2.3. Fish Sexual Development Test (FSDT) exposures with APAP and ASA

Overall, the FSDT experiments followed the OECD test guideline 234 but with smaller differences regarding the experimental design because the experiments were performed before the guideline was validated. These differences are mentioned in the text below. To obtain zebrafish eggs, breeding boxes with artificial plants were put in a breeding tank with parent fish late in the afternoon. The following morning (0 days post fertilization (dpf)) eggs were collected and unfertilized eggs were removed. The fertilized eggs were kept in groups of 100 in 400 mL beakers at 27.5 ± 1 °C for two to three days to minimize embryo and larvae mortality in the tanks during the exposure period, and every morning the undeveloped embryos were removed and substituted with embryos from a reservoir. A total of 40 (APAP FSDT) or 80 (ASA FSDT) embryos were added to each tank (Table 1). At the age of 4 dph (= 7 dpf) larvae were fed once or twice daily with Sera Micron powdered food for fry (Heisenberg, Germany) and at 7 dph this was supplemented with freeze dried decapsulated *Artemia* eggs. At 9 dph the dry food was supplemented with newly hatched *Artemia* sp. nauplii (Inter Yyba GmbH, Germany) once a day. From 20 dph (APAP FSDT) or 15 dph (ASA FSDT) the juvenile fish were fed twice daily with a combination of crushed TetraMin® flakes (Tetra GmbH, Melle, Germany) and decapsulated *Artemia* eggs and once a day with *Artemia* sp. nauplii. The exposure periods were from 2 dpf to 74–75 dph (APAP FSDT) and 3 dpf to 60 dph (ASA FSDT), respectively (Table 2). Triplicate tanks were used for each of the FSDT exposure concentrations.

2.4. Adult exposure with APAP and ASA

Groups of eight adult males and eight adult females were exposed to ASA or APAP for 14 days, and groups of ten males were exposed to ASA for eight days. The exposure concentrations are shown in Table 3.

2.5. Quantification of APAP and ASA in water samples

A one-week pilot study with zebrafish larvae exposed to ASA (1, 10 and 100 mg L^{-1}) and APAP (1, 10 and 100 mg L^{-1}) showed that only 100 mg ASA/L had an effect on mortality (data not shown). Based on the results from the pilot study and a literature survey the nominal exposure concentrations in the experiments shown in Table 1 were selected. These concentrations were chosen to investigate potential