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Inhibition of retinoic acid synthesis disrupts spermatogenesis and fecundity in zebrafish

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ABSTRACT

Timing of germ cell entry into meiosis is sexually dimorphic in mammals. However it was recently shown that germ cells initiate meiosis at the same time in male and female zebrafish. Retinoic acid (RA) has been shown to be critical for mammalian spermatogenesis. Inhibition of RA synthesis by WIN 18,446 has been reported to inhibit spermatogenesis in a wide variety of animals including humans and was once used as a contraceptive in humans. In this study we explored the role of RA in zebrafish spermatogenesis. *In silico* analysis with Internal coordinate mechanics docking software showed that WIN 18,446 can bind to the rat, human and zebrafish Aldh1a2 catalytic domain with equivalent potency. RA exposure resulted in up-regulation of the RA metabolizing enzyme genes *cyp26a1*, *cyp26b1* and *cyp26c1 in vitro* and *in vivo*. Exposure to WIN 18,446 resulted in down-regulation of Aldh1a2, *cyp26a1* and *cyp26b1 in vivo*. WIN 18,446 was effective in disrupting spermatogenesis and fecundity in zebrafish but the reduction in sperm count and fecundity was only observed when zebrafish were maintained on a strict *Artemia* nauplii diet which is known to contain low levels of vitamin A. This study shows that RA is involved in spermatogenesis as well as oocyte development in zebrafish. As the zebrafish Aldh1a2 structure and function is similar to the mammalian counterpart, Aldh1a2 inhibitor screening using zebrafish as a model system may be beneficial in the discovery and development of new and safe contraceptives for humans.

1. Introduction

The primordial germ cells (PGCs) migrate to the undifferentiated gonad where they subsequently, and in response to different factors including retinoic acid (RA), undergo meiosis to initiate gametogenesis (Adams and McLaren, 2002; Koubova et al., 2006; McLaren, 2003). While the timing of germ cell entry into meiosis is sexually dimorphic in mammals, a recent study showed that this does not appear to be the case in zebrafish (Rodriguez-Mari et al., 2013). RA is a determining factor in the timing of meiosis, and initiates ovarian germ cell meiosis during the fetal stage, while on the other hand, testicular germ cells are impeded from entering meiosis until puberty (Bowles et al., 2006; Kashimada et al., 2011). The RA degrading enzyme cytochrome P450, family 26, subfamily b, polypeptide 1 (CYP26B1) show differential expression in the gonads with higher expression in the testis (Bowles et al., 2006; Kipp et al., 2011; Kashimada et al., 2011) and is crucial for inhibiting testicular germ cell entry into meiosis (Koubova et al., 2006). With the onset of puberty, there is a RA surge in the testis that is important for initiation of spermatogenesis (Hogarth and Griswold, 2010). Both vitamin A and its active metabolite RA have been shown to be involved in spermatogenesis. In vitamin A deficient (VAD) rodents, spermatogenesis is impaired and there is a loss of differentiated germ cells (Mitranond et al., 1979; Thompson et al., 1964; Unni et al., 1983). In VAD rat testis only sertoli cells, spermatogonia and spermatocytes were detected (Thompson et al., 1964; Unni et al., 1983). While spermatogenesis and germ cells were restored in VAD rats following dietary retinol addition, RA treatment did not result in a complete recovery (Thompson et al., 1964).

Vitamin A actions in mammalian cells are regulated by different transport proteins, receptors and metabolic enzymes (Griswold et al., 1989). Vitamin A or retinol is transported to tissues by serum proteins known as retinol binding protein (RBP), and enters cells with the aid of the receptor protein stimulated by retinoic acid 6 (Stra6) (Kawaguchi et al., 2007). Once inside the cell, retinol binds to cellular retinol binding protein (CRBP), which then either is esterified and stored as retinyl ester or metabolized to RA. Unlike other signaling molecules such as fibroblast growth factor, transcription factor beta, WNT or hedgehog which binds to surface receptors for signal transduction, RA is lipophilic and enters the nucleus to trigger gene transcription (Duester, 2008). RA binds to cellular retinoic acid binding protein (CRABP) and is transported

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to the nucleus where it binds to nuclear receptors, the RA receptors (RARs) and the retinoid X receptors (RXRs). The RA–RAR–RXR complex then binds to RA response elements on DNA (RAREs) to initiate gene expression (Chambon, 1996). The RARE is generally composed of two direct repeats of consensus sequences RKTTCA or RGKTCA with inter-half-site spacing of 1–5 bp, also known as DR1–DR5 (Mangelsdorf and Evans, 1995; Umesono et al., 1991). The spacing, difference in the hexad half-site sequence and polarity or occupancy of RXR–RAR heterodimer on 5′ and 3′ half-sites can result in different magnitudes of activation (Mangelsdorf and Evans, 1995; Nakshatri and Bhat-Nakshatri, 1998).

Aldehyde dehydrogenase (ALDH1A2) is involved in the synthesis of RA from retinol and WIN 18,446, an inhibitor of ALDH1A2, has been reported to be a potential male contraceptive (Amory et al., 2011). WIN 18,446 belongs to a class of compounds known as bis-(dichloroacetyl)-diamines (BDADs) that inhibit aldehyde dehydrogenases and block RA synthesis (Hogarth et al., 2011). The inhibitory role of WIN 18,446 in spermatogenesis was first noted in rodents (Coulston et al., 1960) and later demonstrated in humans to be reversible and to lack side effects (Heller et al., 1961). WIN 18,446 has been shown to inhibit spermatogenesis without altering the testosterone levels suggesting that its mechanism of action is not hormonal in nature (Amory et al., 2011). Although WIN 18,446 proved to be an effective contraceptive without any side effects, its use in humans was abandoned due to 'disulfiram reaction' after taking alcohol (Amory et al., 2011). Disulfiram (Antabuse) has been used to treat alcoholism (Suh et al., 2006). People taking this medication do not show any symptoms but alcohol intake results in unpleasant physiological responses like flushing, diaphoresis, dysphoria, nausea and vomiting. Disulfiram inhibits liver dehydrogenase-2 (ALDH2) and as a result acetaldehyde, the main metabolite of alcohol builds up and gives the unpleasant reaction (Amory et al., 2011; Suh et al., 2006).

In this study we analyzed the role of RA in spermatogenesis in zebrafish. We investigated if WIN 18,446 as reported in mammals would impair spermatogenesis in zebrafish. RA is reported to be involved in rodent reproduction (Thompson et al., 1964) and therefore fecundity was analyzed following treatment of female zebrafish with WIN 18,446. The results from the present study suggest that *cyp26a1* and *cyp26b1* are important RA metabolizing genes while *cyp26c1* may be less effective. WIN 18,446 inhibited spermatogenesis and fecundity when the fish was kept on a low vitamin A diet.

2. Materials and methods

2.1. Zebrafish maintenance and exposure

In this study wild type zebrafish was used. The fish were fed with flake food (Tetrarubin, Germany) and *Artemia* nauplii (Ocean Nutrition, Belgium). Zebrafish maintenance and exposure was performed as described earlier (Pradhan et al., 2012). For exposure studies, the juveniles at 15 dpf or 20 dpf were exposed to 0.1 and 1 μM of RA (Sigma, USA) and 1 and 10 μM of WIN 18,446 (Carbone Scientific, UK). This time period was selected as zebrafish initiate gonadal differentiation at around 15–20 dpf stage (Takahashi, 1977). WIN 18,446 doses were chosen on the basis of reported IC_{50} value (3 μM) and 100% inhibition (10 μM) of retinoic acid synthesis in H1229 cells (Amory et al., 2011). Both compounds were dissolved in water and added to the exposure beakers to 100 ml final volume. Fifty percent of the water was changed on alternate days and the compounds were replenished to maintain the desired exposure concentrations. Zebrafish juveniles were sacrificed by snap freezing in liquid nitrogen and stored in −80 °C until further use. The use of experimental animals was approved by the Swedish Ethical Committee in Linköping (Permit 32-10).

2.2. Fertilization success and fecundity

Adult male and female zebrafish were kept separately and every 10 days spawning was performed and the number of fertilized and unfertilized eggs was recorded. After 3–4 spawning's, male zebrafish from one pair and female zebrafish from another pair was exposed to WIN 18,446 (10 μM) for 10 days in separate containers. Hundred percent of the water was changed every alternate day. On the 11th day male and female fish were transferred to clean water for mating and the following day fecundity was recorded. After spawning, the male fish was again exposed to WIN 18,446 for next 10 days followed by fecundity analysis. This procedure was repeated 5 times with 6 pairs of fish. In another experiment the dietary intake of vitamin A was limited by feeding the treatment and the control groups with *Artemia* nauplii only, as these have low vitamin A content (Saele et al., 2003).

2.3. Sperm count

After exposure zebrafish were weighed and testes were isolated and weighed in tarred 1.5 mL microcentrifuge tubes using the Precisa 410 AM-FR balance (Precisa Instruments Ltd., Switzerland). Testis samples were submerged in 200 μl Hank's balanced salt solution (HBSS) and crushed using a pestle. Samples were loaded into a 0.1 mm depth haemocytometer (Marienfeld, Germany) and left standing for 3–5 min to allow the sperm to settle. For each sample 5 squares, in the corners and the middle, was counted using a microscope (20× magnification).

2.4. Cell based experiments

ZFL cells (ATCC) were established from adult zebrafish livers and exhibits characteristic of liver parenchymal cells. The ZFL cells were maintained at 28 °C and 3% CO_2 in a humidified incubator in a complex media containing 50% L15 (Gibco, USA), 35% DMEM-high glucose (PAA Laboratories, Austria) and 15% Ham's F-12 (Gibco, USA) supplemented with 5% fetal bovine serum (Hyclone, UK), 15 mM Hepes buffer (Invitrogen, USA), 0.15 g/L sodium bicarbonate (Biochrom AG, Germany). For qRT-PCR analysis, cells were plated (2×10^5 cells/well) in 12 well plates (BD Falcon, USA). The cells were incubated for 16–18 h prior to exposure to RA (0.1 and 1 μM) for 24 h at 28 °C and 3% CO_2.

2.5. Ex-vivo

Adult zebrafish were euthanized and surface sterilized with 70% ethanol. The testes were isolated, transferred to 24 well plates (BD Falcon, USA) and maintained at 28 °C and 3% CO_2 in a humidified incubator in ZFL cell media containing antibiotic–antimycotic solution (Gibco, USA). The explants from the same individual were cultured in parallel; with one testis serving as a control while the other was used for treatment. The testes were exposed to 1 μM of RA and WIN 18,446 for 24 h and further processed for qRT-PCR and *in situ* hybridization.

2.6. RNA extraction and quantitative RT-PCR (qRT-PCR)

RNA extraction was performed using NucleoSpin RNA II kit (Macherey-Nagel, Germany) and qRT-PCR analysis was performed with the primers (Levi et al., 2012; Pradhan et al., 2013) as described previously (Pradhan and Olsson, 2014). In brief, cDNA synthesis was performed using qScript cDNA synthesis kit (Quanta Biosciences, USA). SYBR Green (Kapa Biosystems, USA) was used to determine the expression levels of all genes. Thermocycling conditions for SYBR Green consisted of a denaturation step for 5 min at 95 °C followed by 40 cycles of 95 °C for 2 s and 60 °C for 30 s.