University of Kentucky
UKnowledge

Pharmaceutical Sciences Faculty Publications

Pharmaceutical Sciences

9-15-2019

Imatinib Mesylate Effects on Zebrafish Reproductive Success: Gonadal Development, Gamete Quality, Fertility, Embryo-Larvae Viability and Development, and Related Genes

Nader Ahmadi
Urmia University, Iran

Seyed-Mohammadreza Samaee
Urmia University, Iran

Robert A. Yokel
University of Kentucky, ryokel@email.uky.edu

Aliasghar Tehrani
Urmia University, Iran

Right click to open a feedback form in a new tab to let us know how this document benefits you.

Follow this and additional works at: https://uknowledge.uky.edu/ps_facpub

Part of the [Food Science Commons](#), and the [Pharmacy and Pharmaceutical Sciences Commons](#)

Imatinib Mesylate Effects on Zebrafish Reproductive Success: Gonadal Development, Gamete Quality, Fertility, Embryo-Larvae Viability and Development, and Related Genes

Digital Object Identifier (DOI)

<https://doi.org/10.1016/j.taap.2019.114645>

Notes/Citation Information

Published in *Toxicology and Applied Pharmacology*, v. 379, 114645.

© 2019 Elsevier Inc.

© 2019. This manuscript version is made available under the CC-BY-NC-ND 4.0 license

<https://creativecommons.org/licenses/by-nc-nd/4.0/>.

The document available for download is the authors' post-peer-review final draft of the article.

Imatinib mesylate effects on zebrafish reproductive success: gonadal development, gamete quality, fertility, embryo-larvae viability and development, and related genes

Nader Ahmadi¹, Seyed-Mohammadreza Samaee^{1*}, Robert A. Yokel², Aliasghar Tehrani³

¹Aquatic Lab, Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University, Urmia 165, Iran

²Department of Pharmaceutical Sciences, University of Kentucky, Lexington, KY, USA

³Department of Pathobiology, Faculty of Veterinary Medicine, Urmia University, Urmia 165, Iran

***Author of correspondence:** Seyed-Mohammadreza Samaee; **Tel:** +98-044-32770508; **E-mail:** seyedmohammadreza.samaee@gmail.com & mohammadreza_samaee@yahoo.com; **ORCID:** 0000-0002-9104-0299.

Running title: Imatinib-induced reproductive defects in zebrafish

Abstract – Imatinib (IM) is a tyrosine kinase (TK) inhibitor (TKI) used to treat chronic myeloid leukemia. Clinical case reports and a few laboratory mammal studies provide inconclusive evidence about its deleterious effects on reproduction. The aim of the current study was to evaluate the potential of zebrafish to characterize IM-induced effects on reproduction and clarify IM effects on reproductive success. To this end, we exposed adult zebrafish to four concentrations of IM for 30 days followed by a 30-day depuration period. IM exposure caused a concentration-dependent, irreversible, suppression of folliculogenesis, reversible decrease in sperm density and motility, decreased fecundity and fertility, but no

significant change in atretic follicle abundance. We also observed IM-induced premature hatching, but no significant change in embryo-larvae survivability. However, we found significant IM-induced morphometric malformations. IM decreased expression of *vegfaa* and *igf2a* (two reproductive-, angiogenic-, and growth-related genes) in testes and ovaries. The results demonstrate IM can induce significant changes in critical reproductive endpoints and zebrafish as a suitable model organism to show effects of IM on reproduction. The findings suggest that TKI effects on reproductive success should be considered.

Keywords: *igf2a*, Imatinib, Reproduction, *vegfaa*, Zebrafish

1. Introduction

Imatinib mesylate (IM) is a tyrosine kinase (TK) inhibitor (TKI) (Loren et al., 2013) developed in the mid-1990s (Heinrich et al., 2000). It was first used in 1998 to treat chronic myeloid leukemia (CML) (Mughal and Schrieber, 2010) and has since become a standard therapeutic agent against CML (Ault et al., 2006). IM is the first in a family of highly effective, minimally toxic, agents widely used to treat CML (Zamah et al., 2011).

IM is not specific for Bcr-Abl (a gene often found in CML patients that codes for a TK protein) and binds to at least 25 kinases with varying affinities (Karaman et al., 2008), e.g. KIT, platelet-derived growth factor receptor (PDGFR), abelson-related gene (ARG), and Colony-stimulating factor-1 receptor (c-FMS). These proteins are known to have functions that may play a role in gonadal development, embryo implantation, and fetal development (Tybulewicz et al., 1991; Mauduit et al., 1999; Soriano, 1994, 1997; Hoch and Soriano, 2003). Proteomic analysis of IM-treated human ovarian cancer cells by Patel et al. (2008) showed that exposure of ovarian cells to IM produces complex intracellular changes. Cytotoxicity and genotoxicity of IM for zebrafish has been reported by Novak et al. (2017).

In early clinical case reports exposure to IM was associated with human ovary deficiencies, e.g. a greatly diminished follicle count (Christopoulos et al., 2008), a severely compromised ovarian response to gonadotropin stimulation, and compromised ovarian hormone production and oocyte recovery (Zamah et al., 2011). Results showed decreased numbers of primary follicles and developed Graafian follicles in rats (Yaghmaei et al., 2009b), increased number of pyknotic bodies in mice (Kerr et al., 2012), and increased apoptosis rate in human oocytes (Bildik et al., 2016).

In contrast, there are human clinical case reports, e.g. Malozowski et al. (2008) and reviewed in Hensley and Ford (2003) as well as studies with laboratory animals (Schultheis et al., 2012), in which IM exposure was not correlated with gonadal defect. There are also controversial clinical case reports on IM congenital effects. Some were reviewed by Hensley and Ford (2003), Ali et al. (2006), and Apperley (2009). The above reports provide inconclusive evidence about deleterious effects of IM on reproduction. There is variation among the studies concerning species, age, maturation status, gender, and exposure regimen (e.g. dose, and exposure duration) that most likely contribute to the varying reported observations. Christopoulos et al. (2008) suggested that awareness of the potential for reproductive toxicity will enable physicians to offer patients appropriate counseling and to consider strategies to preserve fertility before prescribing IM chemotherapy.

Zebrafish are the third most frequently used laboratory animal (Goldsmith and Solari, 2003) in a variety of biomedical research fields (e.g., toxicology, drug discovery, and fertility) (Hoo et al., 2016). The use of zebrafish is consistent with the ‘3Rs’ (reduce, refine and replace) approach of animal use for scientific purposes, potentially replacing animals such as mammals with vertebrates that are considered lower on the phylogenetic scale ([The Guide, 2011](#)).

The aims of this study were to analyze adverse effects of IM on 1) zebrafish ovarian follicle development, histopathology, and fecundity, 2) sperm density and motility; 3) viability parameters (fertility, embryo hatchability, and embryo-larvae survivability), 4) larvae morphologic and morphometric malformations, 5) the dose-response of IM-induced reproductive symptoms and their potential reversibility, and 6) the expression of vascular endothelial growth factor Aa [*vegfaa*] and insulin-like growth factor 2a [*igf2a*].

Gonadal development is a complicated process in reproduction in which various genes, biochemical and signaling pathways, and hormonal axes (endocrine, paracrine, and autocrine) are involved. Therefore, selection of specific genes that might explain IM-induced changes in the current study aims was an important challenge. *Vegfaa* and *igf2a* (as angiogenic-, reproductive- and growth-related genes) were selected. Inhibitory effects of IM on *vegfa* (Legros et al., 2004; Raimondi et al., 2014) and *igf2* (Pink et al., 2005; Belinsky et al., 2008; de Waal, 2009) have been reported.

2. Materials and methods

2.1. Fish culture

Wild-type zebrafish (6 months) were purchased from a local supplier in the North of Iran. The fish have been raised through multiple generations within our facility. Care was taken to select zebrafish with continuously high egg production (King Heiden et al., 2006) and fertilization rate (Braunbeck and Lammer, 2006). For fish housing and maintenance see Samaee et al. (2015). Zebrafish were used and cared for in accordance with standard protocols and guides (Westerfield, 2007; Guide for the Care and Use of Laboratory Animals, 8th edition, 2011).

2.2. Preparation of IM suspension

IM (Gleevec®; 100 mg/tablet) was obtained from Osvah Pharmaceutical Co. (Tehran, Iran). Two tablets were ground using a mortar and pestle and the resulting powder was added to 500 mL of 2.5% NaCl [Merck] solution yielding 0.4 mg mL⁻¹ IM. The suspension was sonicated (power: 70%; duty: 50%) for 30 min (resulting in a homogeneous cloudy suspension). The IM dose was based on the average human dose of 600 mg/day to control CML (Ohyashiki et al., 2002; James et al., 2003; Prabhash et al., 2005; Christopoulos et al. 2008; Pye et al., 2008; Zamah et al., 2011; Bay et al., 2013), equal to 8 mg per kg in a human of 75 kg. Fifty mL IM-BSN suspension (0.4 mg IM mL⁻¹ [see section “2.5”]) was added to 1950 mL tank water (see section “2.6”) providing fish with a nominal concentration of 10 µg IM mL⁻¹.

To ascertain the effects of IM tablet excipients a mix of all the excipients minus the active substance was obtained from Osvah Pharmaceutical Co. Zebrafish were exposed to the excipients in 3 replicates of 10 zebrafish. Endpoints such as the abundance of follicular stages, fecundity, and sperm density and motility were considered to evaluate the biological effects of the excipients.

2.3. Production of brine shrimp nauplii (BSN) feed for the zebrafish

BSN were prepared using the cyst of *Artemia franciscana*, strain VC, obtained from Can Tho University. The cysts were cultured, hatched overnight, harvested, cleaned, and resuspended as described (Varga, 2011). The total number of BSN in 500 mL cleaned hatching mixture averaged 94,000.

2.4. Preparation of “BSN suspension”

A determined number of harvested BSNs were washed in a 100 mL glass cylinder with 2.5% salt water, adjusted to 100 mL with 2.5% salt water, and the “BSN suspension” stored at 4 °C with moderate aeration until use.

2.5. Preparation of “IM-BSN suspension”

A determined number of harvested BSNs were washed in a 500 mL glass cylinder with 0.4 mg mL⁻¹ “IM suspension” (see section 2.2), adjusted with “IM suspension” to 500 mL, and the “IM-BSN suspension” moderately and continuously aerated at 28 °C for less than 1 hour.

The *Artemia* nauplii used to prepare the IM-BSN suspension were newly hatched that grow on their yolk sac, don’t externally feed, and therefore don’t take up IM (Van Stappen, 1996), obviating drug metabolism. BSN is a live attractive food for zebrafish. When zebrafish feed on BSN they also ingest the IM suspension. This approach differs from most adult zebrafish drug delivery studies that used direct addition to water (e.g. Maley et al., 2013; Samaee, 2015; Dr. Kiranam Chatti, University of Hyderabad, India [2018], personal communication).

2.6. IM exposure procedure

Zebrafish were housed in 5 L mouse Plexiglas cages (Razirad Co, Iran) Samaee et al. (2015). Treatment groups were defined by the frequency of zebrafish feeding with “IM-BSN suspension” and “BSN suspension” for 1 month as follows:

“IM-0” (control group): 3 feedings per day with BSN only (1, 5, and 9 h after light on). “IM-1”: received a single (1 h after light on) IM-BSN feeding, and 2 regular BSN feedings. “IM-2”: received 2 IM-BSN feedings and 1 BSN feeding. “IM-3”: received 3 IM-

BSN feedings per day. Each treatment was performed in 3 replicates (with a total of 20 zebrafish per replicate).

For feedings, the tank filter unit was switched off and some tank water discarded so that 1950 mL water remained per tank. Fifty mL of BSN suspension was added (with or without IM). We allowed fish 1 h (Maley et al., 2013) to consume nauplii, thereafter the tank water was completely changed.

IM-induced reproductive endpoint changes were evaluated at two time points: 1) at the end of the IM exposure period, and 2) after 1 month of IM depuration (to assess recovery from IM exposure effects).

2.7. Validation of IM presence in zebrafish tissue

IM-treated fish brains were collected and extracted according to Zhang et al. (2016). Analyses were performed with Waters Alliance 2695 HPLC-Micromass Quattro micro API Mass Spectrometer. LC conditions – mobile phase: MeOH + 0.1% Formic Acid and H₂O, column: Atlantis T3-C18 3 μ , 2.1 $\times$ 100 mm, column temperature: 30 °C, flow rate: 0.2 mL/min, and injection volume: 10 μ L. MASS conditions – mode: ESI+, cone Volt: 45 V, capillary Volt: 3 kV, extractor: 3 V, RF lens: 0.2 V, collision Energy: 35 eV, gas nebulizer: N₂ (grade 5), flow gas: 200 L/h, source temperature: 150 °C, and desolvation temperature: 350 °C.

2.8. Ovarian histology and morphology

Fish were collected after one month of IM exposure and 1 month after IM treatment was stopped. At least six females (King Heiden et al., 2006) from each treatment and control group were fasted (for 24 h) then euthanized (Gupta and Mullins, 2010). The fish were decapitated, tails removed, an incision made along their ventral surface, and fixed in 10%

formaldehyde (Samaee et al., 2009). Fixed specimens were decalcified by placing them in a decalcifying buffer (5 mL formic acid, 5 mL formaldehyde, and 90 mL dH₂O) for one week at room temperature. Specimens were dehydrated in a graded series of ethanol, cleared in xylene, and embedded in paraffin. For each specimen, 4-6 distinct sagittal sections at 5 μ m (taken every 20 μ m) from the same region, were transferred onto microscopy slides, stained with hematoxylin and eosin, and cover slipped. Sectioning was standardized according to the relative position of the organ of interest to other structures (Fig. 1b). Sections were examined under a light microscope (C82612, Olympus, Japan) equipped with a digital camera (CMEX DC. 1300, Netherlands) at 100 $\times$ and 400 $\times$ magnification.

Ovarian follicles were counted and assigned their appropriate gametogenic stage (Fig. 2), based on morphological characteristics as defined by Selman et al. (1993) and Silva et al. (2012). The relative number of follicles at each of the five gametogenic stages/total follicles counted $\times$ 100 was calculated (= frequency), based on more than 100 follicles in each ovary (Wang et al., 2011). Images were recorded at 100 $\times$ and 400 $\times$ magnification. Ovarian follicle diameter was determined on digital photomicrographs using Image Focus (V. 13, Euromex Microscopen b.v., 2005-2006). For each specimen and each follicular stage, we determined the diameter of 10-12 follicles. The identification of atretic follicles and determination of their atresia level (the degeneration of ovarian follicles that do not ovulate), was performed according to Miranda et al. (1999) after discussing with several fish histopathologists.

2.9. Sperm analysis

Fish were fasted for 24 h (Kinkel et al., 2010), anesthetized with cloves (see Grush et al., 2004; half teaspoon of blended dried flowering clove buds, *Eugenia caryophyllata*, in 250 mL of system water for $\sim$ 1 min), euthanized, rinsed in fresh water, blotted, weighed (Yang et al., 2007), and immersed in Hank's balanced salt solution (HBSS) (Hagedorn and Carter,

2011) (in a ratio of $\sim 1:50$ [mass:volume]) (Dr. Huiping Yang, LSU AgCenter, personal communication). The testes were minced in HBSS (Ingermann et al., 2011) with fine scissors. Testes pieces were gently aspirated into and ejected from an Eppendorf pipette > 20 times to disrupt the tissue and to release mature sperm cells. Large testes fragments were removed with fine forceps, or by a 30 s spin to isolate heavy tissue fragments from the supernatant suspension (Dr. Qiaoxiang Dong, Institute of Watershed Science and Environmental Ecology, China, personal communication). The resulting suspension containing sperm can be held on ice for ≤ 2 h (Westerfield, 2000; Hagedorn and Carter, 2011).

Sperm density was determined using a hemocytometer. A bright field microscope (Eclipse E600, Nikon, Japan) equipped with a digital camera (Cyber-shot DSC-W130 8.1MP Digital Camera with 4x Optical Zoom with Super Steady Shot, Sony, Japan) was used to record sperm motility. The recordings were analyzed using EDIUS 6 and the percentage of motile sperm within 5, 10, 15, 20, 25, and 30 s after activation, start of sperm movement after adding water, (denoted spa [seconds post activation]) determined.

2.10. Breeding, embryo collection, and fecundity and fertility determination

Fish breeding, embryo collection and culture, determination of fecundity, embryo/larvae viability parameters (fertilization rate [FR], hatching rate [HR], and survivability rate [SR]), and recording of morphometric characteristics (MC) were carried out according to Samace et al. (2019). For the breeding trials, 16 zebrafish (8 males and 8 females) were taken from the treated (IM-3) and 16 from the untreated (IM-0) groups (Fig. 1c-f). To further investigate the effects of IM in both sexes, male and female replacement experiments were conducted. For the male replacement trial eight untreated males were

housed with IM-3 treated females [Fig. 1e]). For the female replacement trials, eight untreated females were placed with IM-3 treated males [Fig. 1f]).

2.11. Gene expression analysis

Ovary/testis pieces were snap frozen in liquid nitrogen and stored at -80 °C until use. Six specimens from each treatment group were used for gene expression analysis. Total RNA was extracted using a Bio FACT™ Kit (RP101- 050, Daejeon, Korea) following the manufacturer's instructions, eluted in 20 µL RNase-free water, its quality/quantity determined by spectrometry (NanoDrop-Thermo 2000C; Thermo Fisher Scientific, Wilmington, DE, USA), then stored at -80 °C. Total RNA (10 ng) was reverse transcribed into cDNA using a BioFACT™ Kit (BR441-096, Daejeon, Korea) in a 20 µL reaction volume by a thermal cycler (model, PEQLAB, Germany), cDNA quantity evaluated by spectrometry, and stored at -20 °C.

Two target genes (*vegfaa* and *igf2a*) and a housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase [*gapdh*]), as internal standards for the target genes, were used for gene expression analysis. The primers were designed with Primer Express Software (Applied Biosystems) using identical parameters to generate amplicons of similar size. The primers were synthesized by Metabion International AG (Germany).

Quantitative real-time PCR was performed using the StepOne Plus™ system (Applied Biosystems, Foster City, CA, USA). Each reaction contained 0.5 µL diluted cDNA, 0.05 µL forward (10 µM) and 0.05 µL reverse (10 µM) primers, and 10 µL SYBR Green PCR Master Mix to a final volume of 20 µL. Amplification followed the PCR cycle condition: 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 61 °C. Each treatment was analyzed in triplicate (biological replicates) and each sample in triplicate (methodological replicates). A non-template control was performed to ensure that only one PCR product

amplified and the stock solutions were not contaminated. Cycle conditions and amounts of templates were optimized for each primer set in pilot experiments to ensure that amplification was terminated within the linear phase.

A melting curve was also performed to ensure the specificity of PCR amplification. The melt curve protocol was 15 s at 95 °C once and then 10 s each at 0.3 °C increments between 60 °C for 1 min and 95 °C for 10 min. Data collection was enabled at each increment of the melt curve. The amplification efficiency, specificity of primers, and amount of cDNA/sample were tested by the standard curve method. Primer pairs were deemed to be acceptable for analysis if they generated standard curves with an r^2 value above 0.98, there was consistency among replicates and the primer amplification efficiency was 85–110%.

RNA expression levels are presented as cycle threshold values. The relative expression of genes was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using *gapdh*. The *gapdh* did not change among different treatments in any of the experiments. The expression levels of the *vegfaa* and *igf2a* were evaluated relative to *gapdh*. IM-3 treated fish *igf2a* and *vegfaa* gonad expression level was evaluated relative to untreated fish.

2.12. Statistical analysis

Values are reported as mean $\pm$ S.D. Differences among treatment groups were evaluated by one-way analysis of variance followed by Duncan's multiple range test (post-hoc) for all endpoints. A p-value of 0.05 was used to set statistical significance levels. Simple regression models were formulated between endpoint magnitudes and the IM exposure frequency to identify concentration-dependent IM-related effects. The levels of significance (≤ 0.002 [for gonadal parameters] and $p \leq 0.003$ [for morphometric ratios]) for the regression analyses were selected after applying the Bonferroni's adjustment to minimize the chance of

type I statistical error. All analyses were performed using IBM SPSS (version 20; SPSS Inc., Chicago, IL, USA), and Excel 2010 (Microsoft Corporation, Redmond, WA, USA).

3. Results

3.1. Effects of IM tablet excipients on reproductive endpoints

No significant difference was observed between tablet excipient-treated zebrafish (e.g. abundance of follicular stages [e.g. PN; $11.6 \pm 2.0\%$], fecundity [160.8 ± 23.1], sperm density [$4.2713 \times 10^8 \pm 7.29971969 \times 10^7$ per mL], and sperm motility [e.g. 5 spa; $77.4 \pm 10.3\%$]) and controls (e.g. abundance of follicle stages [e.g. PN; $10.6 \pm 1.4\%$], fecundity [154.8 ± 16.4], sperm density [$4.490750000 \times 10^8 \pm 6.03184000 \times 10^7$ per mL], and sperm motility [e.g. 5 spa; $80.1 \pm 4.0\%$]) concerning the evaluated reproductive endpoints in the current study.

3.2. Presence of IM in tissue

Zebrafish brain IM was 0.08 ± 0.02 ng mg⁻¹. An IM plasma level of > 0.5 µg mL⁻¹ (0.5 ng mg⁻¹) is needed for tyrosine kinase inhibition in vitro (Peng et al., 2005), $> 1,000$ ng mL⁻¹ (1 ng mg⁻¹) to control GIST and CML (Eechoute et al., 2011), and 0.25 µM to achieve 50% inhibition of cellular Bcr/Abl-related tyrosine phosphorylation (Wolff et al., 2003). IM crosses an intact blood–brain barrier poorly (Holdhoff et al., 2010). In the study of Wolff et al. (2003) mice received 150 mg kg⁻¹ IM daily orally. Plasma IM was 6958 ng mL⁻¹, 155 times higher than in the cerebral spinal fluid (CSF) (45 ng mL⁻¹). In Azanza et al. (2018) the IM concentration of CSF was 74 times lower than in the plasma. Therefore, related to the brain IM concentration in the current study the IM level in zebrafish plasma was probably at a clinically-relevant level.

3.3. Frequency of ovarian follicular stages

Ovaries of zebrafish treated 1, 2, or 3 times per day with IM had significantly more follicles at the PN stage than controls (Fig. 3a), but significantly fewer follicles at eCA, mCA, V, and M stages (Fig. 3b to e). The number of ovarian follicles at the PN stage [Fig. 4a] positively correlated and at the eCA, mCA, V, and M stages [Fig. 4b to e] with the daily IM exposure frequency.

We only found a statistically significant difference between IM-treated fish at the end of IM exposure and the depuration period regarding the proportion of follicles at the mCA stage [Fig. 5c]. At the end of depuration there were non-significant trends toward fewer follicles at eCA and V (Fig. 5b and d), and more follicles at PN, mCA, and M (Fig. 5a, c and e) stages.

3.4. Follicle diameter

IM-1, 2, and 3 treated zebrafish ovary follicle diameters at PN, eCA, mCA, V, and M (Fig. 3 f to j) stages were consistently smaller compared to controls (IM-0), in some cases significantly. We found a significant, negative linear relationship between the diameter of follicles at the M stage and the frequency of IM exposure (Fig. 4j). Although a negative linear relationship was also observed for the other follicular stages (PN, eCA, mCA, and V [Fig. 4f to i]), the correlations were not statistically significant.

There were no significant differences between the two time-points regarding the diameter of follicles at gametogenic stages (eCA, mCA, V, and M [Fig. 5g to j]). After the depuration period, treated zebrafish had significantly smaller PN (Fig. 5f), non-significantly smaller eCA, mCA, and V (Fig. 5g to i), and larger M (Fig. 5j) ovarian follicles.

3.5. Fecundity

A significant decrease in fecundity was observed in IM-treated (108 ± 28) zebrafish compared to untreated specimens (control; IM-0, 171 ± 55). The IM-induced decrease in female fecundity did not recover after the 1-month drug depuration period (104 ± 19).

3.6. Sperm density and motility

The highest and lowest sperm densities were observed in IM-0 (control) and IM-3 treated zebrafish, respectively (Fig. 6a). There was a significant and negative linear relationship between zebrafish sperm density and IM exposure frequency (Fig. 7a).

The highest motility was observed in IM-0 and the lowest motility in IM-3 treated fish at all observation times (Fig. 6b-g). There was a significant decline in the percentage of motile sperm with time (i.e. from 5 to 30 spa) in both IM-treated and untreated zebrafish. There was a significant and negative linear relationship between sperm motility and IM exposure frequency (Fig. 7b-g).

The IM-induced decrease in sperm density and motility recovered during the depuration period. At the end of the depuration period sperm cells in IM-1, IM-2, and IM-3 treated fish had a density and motility similar to that of untreated fish (IM-0) (results not shown).

3.7. Viability

3.7.1. Fertility

There was a significant fertility difference between IM-3♀ × IM-3♂ pairs and controls (IM-0♀ × IM-0♂) (Fig. 1c and d). In the male replacement (IM-3♀ × IM-0♂) and female replacement (IM-0♀ × IM-3♂) trials the fertility rate partially recovered (Fig. 1e and f). There was a significant difference between the male replacement (IM-3♀ × IM-0♂) and

control (IM-0♀ × IM-0♂) although such a significant difference was not observed between female replacement (IM-0♀ × IM-3♂) and control.

3.7.2. Embryo hatching rates

The IM-3♀ × IM-3♂ embryos had a significantly higher HR at 48 hpf compared to IM-0♀ × IM-0♂ embryos. A significant difference was not observed in HR at 72 hpf. In the male replacement trial, the IM-3♀ × IM-0♂ embryos had a 48 hpf HR -lower than IM-3♀ × IM-3♂ and higher than IM-0♀ × IM-0♂ embryos. In the female replacement trial, the IM-0♀ × IM-3♂ embryos had a 48 hpf HR lower than IM-3♀ × IM-3♂ and IM-3♀ × IM-0♂ embryos, similar to IM-0♀ × IM-0♂ embryos. We observed no such difference in HR at 72 hpf (Fig. 1c-f).

3.7.3. Embryo-larvae survival rate

We found no significant difference in 24-240 hpf embryo-larvae survivability between IM-3♀ × IM-3♂ (mean ± S.D; 88.2 ± 7.7%) and IM-0♀ × IM-0♂ (mean ± S.D; 89.5 ± 11.0%) progeny. We also did not find any significant difference in 24-240 hpf embryonic or larval survival rates between the offspring of replacement trials (male [IM-3♀ × IM-0♂; 95.8 ± 1.2%] and female [IM-0♀ × IM-3♂; 99.2 ± 1.2%]) and IM-3♀ × IM-3♂ (88.2 ± 7.7%) and IM-0♀ × IM-0♂ (89.5 ± 11%).

3.8. Larvae morphometric alterations

The IM-exposed groups significantly differed from controls for changes in morphometric ratios at different larval developmental stages (Table 1). In Table 1 all ratios but one (row 19) were different for at least one IM treatment group from control. Significant variations were also observed among all but 4 IM-exposed groups (Table 1, rows 11, 15-16,

and 18). The variations of 19 morphometric ratios (as dependent variables) at 72, 96, 120, 144, 168, 192, and 240 hpf were significantly correlated to the frequency of exposure to IM (as the independent variable) by simple regression models as either linear (Table 2, boldface data) or quadratic (Table 2, underlined data) functions.

3.9. IM-induced changes in *vegfaa* and *igf2a* expression

The expression levels of *igf2a* and *vegfaa* were significantly down-regulated in the ovary of IM-3-treated adult zebrafish relative to untreated fish (IM-0), by 2.10- and 2.30-fold, respectively. A decreased expression level of these four endpoints was also observed in the testes of IM-3-treated adult zebrafish relative to untreated fish, by 2.13-fold and 2.30-fold, respectively.

4. Discussion

IM is a class of chemotherapeutic agents with inconclusive reports about its adverse effects on reproduction of humans and laboratory animals. In the current study, for the first time, zebrafish were used as an alternative model to characterize potential IM-induced reproductive effects.

4.1. Effects of IM on ovary development

Exposure to IM caused a delay in zebrafish ovarian follicular development that was indicated by the significantly higher presence of early-stage perinucleolar follicles in all three IM-treated groups and a suppression of size in the IM-3 treated group compared to control. In the study of Asadi-Azarbaijani et al. (2017) the treatment of rats on postnatal days 2–4 with IM (150 mg/kg) led to delay in the formation of primordial follicles, increased density of multi-oocyte follicles, oogonia, and germline clusters, and decreased activation of primordial

follicles. In brief, the follicular defect was attributed to the inhibitory effect of IM on 1) TK signaling pathways, 2) growth factors, 3) transcription factors, and 4) steroidogenic regulatory proteins that are involved in early folliculogenesis. The inhibiting effect of IM on follicle activation in the Asadi-Azarbaijani et al. (2017) study is also supported by a study that showed significantly decreased numbers of primary follicles, developing Graafian follicles, and developed Graafian follicles in adult IM-treated (50 and 100 mg/kg for 14 days) rats (Yaghmaei et al., 2009b).

There are also studies in which the exposure to IM led to a significant decrease in total number of ovarian follicles, e.g. a massive follicle loss was reported in IM-treated (2.5, 5, and 10 μ M for 12, 24, 48 and 72 h) ovaries from humans (Bildik et al., 2016). Yildiz et al. (2016) also cited a significant decrease in the ovarian follicle number in IM-treated (25 mg/kg/day for 14 days) rats.

Christopoulos et al. (2008) reported a single case of a 28-year old woman with CML who developed primary ovarian insufficiency after treatment with 400–600 mg/day IM for 2 years although the report was criticized by Malozowski et al. (2008) as possibly being only a chance finding. In the study of Schultheis et al. (2012) IM (150 mg/kg/day for 2 months) had no effect on murine ovary. There was a normal succession of follicle development from the primordial stage to ovulation in leukemic mice.

In the current study, IM-induced defects in folliculogenesis were concentration-dependent (Fig. 4a-e) and consistent with the study of Bildik et al. (2016) in human ovaries. The overall statistical strength of the association was higher at later follicular stages (V and M [Fig. 4d and e]) compared to earlier stages (PN, eCA, and mCA [Fig. 4a-c]), which can be explained by follicles at advanced maturity stages having a higher probability of being atretic (Silva et al., 2012), and therefore more sensitive to IM than earlier gametogenic stages.

In our study, the exposure to IM led to a decrease of the diameter of PN, mCA, and M follicles, and a non-significant decrease in the diameter of eCA and V follicles, compared to controls, i.e. a decrease in follicle diameters at all gametogenic stages.

Although the smaller follicles in the current study at later gametogenic stages are thought of as a disadvantage for the offspring (Weber et al., 2003), the biological effects of the decrease in the size of follicles at earlier stages (i.e. PN and eCA, Fig. 4f and g) remains unclear.

Cortical alveoli are synthesized endogenously, involving the rough endoplasmic reticulum (ER) and the Golgi complex (Tyler and Sumpter, 1996). Adverse effects of IM on ER function (i.e. the induction of ER stress) have previously been reported (Hu et al., 2012). Thus, the decrease CA follicle size in the ovaries of IM-treated zebrafish compared to control in the current study can be correlated to adverse effects of IM on ER function.

Except for the percent of oocytes in the mCA stage and PN diameter (Fig. 5 c and f) we did not find significant differences between the end of the 30-day IM exposure and after 30 days of depuration regarding follicular development (follicle number [Fig. 5a-b, d-e], and diameter [Fig. 5g-j]) at the different gametogenic stages. This suggests that IM may have irreversible effects on zebrafish folliculogenesis after exposure.

4.2. Sperm density and motility

Zebrafish IM exposure led to a significant concentration-dependent decrease in sperm density and motility (Figs. 6 and 7). This is consistent with adult rat and human studies. Decreased sperm cell number at different gametogenic stages was seen in rats exposed to 6-12 mg/kg for 30 days (Yaghmaei et al., 2009a). Sixty mg/kg of IM for 70 days reduced motile sperm number in rats (Novartis Pharmaceuticals Corporation, 2018). Oligospermia was seen in a human who received 400-800 daily for 5 months – Seshadri et al., 2004) and in

a human who received 400 mg daily for 35 months (Mariani et al., 2011). In contrast, no difference in spermatogenesis was observed in IM-treated and untreated adult mice continuously exposed to IM, 150 mg daily, for 2 months (see Apperley, 2009), and normal sperm production was seen in IM-treated leukemic mice (150 mg/kg/day for 2 months) (Schultheis et al. (2012)). The latter observation was attributed to the redundancy of the tyrosine protein kinase pools in gonads. A lack of IM-induced change in sperm motility was also observed in rats treated with ≤ 20 mg/kg attributed to the low IM dose (Novartis Pharmaceuticals Corporation, 2018).

In the current study, the IM-induced decrease in sperm parameters recovered during the depuration period. This agrees with prior studies. An IM-induced 84% reduction in epididymal sperm in 35-day old mice that received 150 mg/kg IM from postnatal days 1 to 5 returned to normal when 90 days old (Basciani et al., 2008)) and an IM-induced defect in spermatogenesis in immature rats (150 mg/kg on postnatal days 5 to 7) recovered by 11 weeks of age (Nurmio et al., 2007).

4.3. Viability

We observed a significant fertility difference between IM-3♀ $\times$ IM-3♂ pairs and controls. In the male replacement (IM-3♀ $\times$ IM-0♂) and female replacement (IM-0♀ $\times$ IM-3♂) trials, fertility recovered (increased). In contrast, fertility was not affected in female rats which had received less than the maximum clinical IM dose for 14 days before mating and through day 6 of gestation (Novartis Pharmaceuticals Corporation, 2018).

The IM-3♀ $\times$ IM-3♂ subjects had a significantly higher hatching rate at 48 hpf compared to controls (IM-0♀ $\times$ IM-0♂) (55.2 vs, 0%). The lower hatching rate in male replacement trials (IM-3♀ $\times$ IM-0♂) (22.5%) and lack of hatching in female replacement trials (IM-0♀ $\times$ IM-3♂) (0%) at 48 hpf indicates that an effect of IM on eggs contributes to

accelerated hatching. The change in hatching time is an important stress response of fish larvae (Barton, 2002). Hatching is a consequence of hatching enzyme activity and embryo movements (Winnicki et al., 1970; Yamamoto, 1975). Zebrafish tyrosine kinase expression is regulated in the first 24 h of development, suggesting their role during early embryonic development (Challa and Chatti, 2013). The inhibitory role of IM on tyrosine kinases that control hatching events may contribute to the accelerated embryo hatching produced by IM-treated fish.

4.4. IM-induced changes in morphometric ratios

There was significant variation between larvae produced by IM-treated and untreated zebrafish with respect to the morphometric measurements (as a sublethal response) (Table 1). The morphometric alterations were dependent on IM exposure frequency (Table 2).

In comparison to morphological based toxicology assays, morphometric based assays are more relevant to fish larvae growth and developmental response to toxins. The MCs are used to assess impact on growth and development of larvae at exposure concentrations well below those that change larvae survival or produce any remarkable increase in gross malformations, as observed in the current study (Prof. Dr. Jennifer L. Freeman, School of Health Sciences, Purdue University; personal communication).

IM-induced congenital malformations have been reported in animals and humans. Exposure of rats to IM (50 mg/kg/day) led to organogenesis defects, while such teratogenic effects were not observed in rabbits (Novartis investigator's brochure and Novartis clinical safety statement [see Schultheis et al., 2012]). Two of 16 infants had minor abnormalities at, or shortly after, birth (Ault et al. (2006)). A study of 180 pregnancies by Pye et al. (2008) disclosed an IM-induced increase in the risk of fetal malformations.

In the current study three groups of IM-induced morphometric alterations were seen: The first group was present at early larval stages (BL/APB [Table 1, row 7], BL/BD2 [Table 1, rows 8, and 13], PoPB/BD2 [Table 1, row 10], BD2/HL [Table 1, row 11], BD1/BD2 [Table 1, row 15], and BD1/HL [Table 1, row 16]). The alterations disappeared at advanced stages. The second group occurred only at later larval stages (APB/HL [Table 1, row 19]). The third group was found to be stable through all larval stages (BL/BD1 [Table 1, rows 1, 3, 6, 12, 18, 20, 23, and 25], APB/BD1 [Table 1, rows 2, 4, and 21], APB/BD2 [Table 1, rows 5, and 22], BL/HL [Table 1, rows 9, and 24], and PoPB/BD1 [Table 1, rows 14, and 26]).

The above-mentioned ratios were created from MCs related to body length (BL, HL, APB, and PoPB) and body depth (BD1 and BD2 [i.e. yolk depth/size]). Such MCs of zebrafish larvae have also been considered to characterize the toxicity of other agents (e.g. George et al., 2011; Brannen et al., 2010; Wirbisky et al., 2016).

The second and third groups of morphometric ratio changes show the potential to characterize IM-induced responses. In general, a key element complicating the establishment of a link between exposure and a health defect is the time that elapses between exposure and response (Gluckman et al., 2008; Barouki et al., 2012). It may take years for an individual to present a health defect and pass it on to future generations (Jirtle and Skinner, 2007). In zebrafish the morphometric changes that appeared within a short period of exposure to different IM concentrations could predict later-in-life consequences of IM embryonic exposure.

4.5. Changes in *igf2a* and *vegfa* expression

Exposure of female zebrafish to IM led to retardation at the transition of follicles from the PN to the eCA stage (Fig. 3). These types of defects in ovary development have been reported in zebrafish treated with *igf2a*-inhibitors such as difenoconazole (Teng et al., 2017).

igf2a is dynamically expressed during folliculogenesis in zebrafish. Its expression starts to increase from the PN stage (> 0.1 mm), reaching its highest level in the eCA stage (~ 0.30 mm) and then decreases (Li et al., 2015). Inhibitory effects of IM on the human *igf2a* homologue (*igf2*) have been reported (Pink et al., 2005; Belinsky et al., 2008; de Waal, 2009). The concurrent incidence of the transition retardation of ovarian follicles from the PN to the eCA stage with ovarian *igf2a* down-regulation in the IM treated zebrafish suggests a role of *igf2a* in the defect.

In the current study, a decrease (~ 6 -fold) was observed in the abundance of ovarian follicles at the V stage, i.e. a developmental block in follicular maturation to stage V (Fig. 3d). Recombinant zebrafish *igf2a* proteins directly enhance oocyte maturation via *igf1* receptors (Li et al., 2015). In the zebrafish study of Nelson and Van Der Kraak (2010), recombinant human *igf2* stimulated germinal vesicle breakdown in early vitellogenic (0.35–0.44 mm), midvitellogenic (0.45–0.56 mm), and full grown (0.57–0.65 mm) follicles incubated *in vitro*. *igf2a* also mediates luteinizing hormone (LH) action on oocyte maturation in zebrafish (Li et al., 2015). These observations may be attributable to either a direct effect of the IM-induced *igf2a* decrease on developing follicles or to an indirect disruption of the pituitary-gonadal endocrine system. Endocrine-related side effects of TKIs, e.g. alterations in thyroid function, bone metabolism, linear growth, gonadal function, fetal development, glucose metabolism, and adrenal function were reviewed by Lodish et al. (2010; 2013). The effects of IM on different parts of the reproductive endocrine axis in males and females have been seen, e.g. on progesterone, estrogen, follicle stimulating hormone (FSH), and LH concentration in the rat (Yaghmaei et al., 2009a and b), inhibin-B/FSH ratio in the human (Mariani et al., 2008), and testosterone in the human (Gambacorti-Passerini et al., 2003).

An IM exposure frequency-dependent decrease in follicle diameters was found at all follicle stages (Figs. 3f-j and 3f-j). The vascular network is essential for providing organs

with oxygen, nutrients, and hormones, as well as removing metabolic waste products (Haigh, 2008; Kim et al., 2013). The *veg*f gene family provides required signals for vasculogenesis (*de novo* formation of blood vessels) during embryogenesis and for angiogenesis (formation of new blood vessels from preexisting vessels) during organogenesis (Kim et al., 2013). Bahary et al. (2007) isolated a duplicate *vegfa* locus in zebrafish (*vegfaa* and *vegfab*). *vegfa* expression has been correlated with the proliferation of blood vessels in the ovary in mammals (Phillips et al., 1990; Ravindranath et al., 1992; Kaczmarek et al., 2005). Folliculogenesis is dependent on new capillary vessel proliferation (Ferrara, 2004). McFee et al. (2009) and Artac et al. (2009) cited the involvement of *vegfa* in rat folliculogenesis. *vegfa* production in the ovary is critical for normal reproductive function in mammals (Geva and Jaffe, 2000). Oocyte size in adult fish at later ovary stages is determined by the uptake of systemic circulating vitellogenin (Vtg) protein. Vtg is synthesized and secreted by hepatocytes, transported to the ovary via blood, and finally deposited in oocytes as Vtg-derived egg yolk proteins (Wallace, 1985; Arukwe and Goksøyr, 2003). Yolk proteins play a key biological role during embryogenesis and larvae development. The decrease in follicle size s in all follicular stages in IM-treated fish may be attributed to the reduction in angiogenesis (because of *vegfaa down-regulation*) that led to a decrease in availability of Vtg and maybe endocrine regulators.

igf2 is also involved in angiogenesis (Herr et al., 2003), exerting its angiogenic effects both directly and via a *vegfa* mediated process (Belinsky et al., 2008).

In the current study, a significant decrease in fecundity was observed in IM-treated zebrafish (section 3.5). In the study of Zhu et al. (2015) the number of produced eggs in zebrafish positively correlated with the level of ovary *igf2a* expression. *igf2a* is dynamically expressed during folliculogenesis in zebrafish (Li et al., 2015). Wang et al. (2011) suggested that

reduced egg production may reflect a defect in folliculogenesis. Therefore, the decreased fecundity observed here can be attributed to the IM-induced reduction in *igf2a* mRNA.

Exposure of adult male zebrafish to IM led to an exposure frequency-dependent decrease in sperm density (Figs. 6a and 7a). The presence of *igf2a* in zebrafish testes has been cited by Irwin (2011), Nóbrega et al. (2015), Safian et al. (2016), and Teng et al. (2017). *igf2* has a selective paracrine or autocrine role in the regulation of spermatogonial proliferation during spermatogenesis (Gupta, 2005). In humans *igf2* promotes the differentiation of spermatogonia into primary spermatocytes (Nakayama et al., 1999). The significant decrease in sperm density in IM-treated zebrafish may be attributed to the reduction in spermatogonial proliferation and differentiation due to testis *igf2a* down-regulation.

IM-treated zebrafish had a lower number of motile sperm than (controls) (Fig. 6b-g). *vegfa* is present during testis morphogenesis and expressed by Sertoli cells which play an important role in testis formation and function in the human (Funke et al., 2010). Alterations in *vegfa* expression have frequently been correlated to testis developmental and functional impairments in the mouse (Korpelainen et al., 1998); rat (Bott et al., 2006; Baltes-Breitwisch et al., 2010) and human (Funke et al., 2010). The *vegf* protein is present in human spermatids, Sertoli and Leydig cells, and seminal plasma (Obermair et al., 1999). *vegf* has an important role in human and murine sperm physiology, maturation, and fertility (Obermair et al., 1999; Nalbandian et al., 2003). The effects of the protein on human sperm motility (Iyibozkurt, 2008) and survival (Attar et al., 2005) have been cited. The inhibitory effect of IM on *vegf* expression was cited in mammals (Legros et al., 2004; Raimondi et al., 2014). The concurrent reduction in the number of motile sperm and *vegfaa* expression in IM-treated zebrafish testis in the current study suggest *vegfaa* down-regulation can be considered as one of reasons for sperm success failure.

Although we did not find a significant difference between IM-treated and untreated zebrafish concerning embryo-larvae survival in the current study (see section 3.7.3), we found significant MC ratio differences (Table 1). It is clearly evident that *igf2* is important during post-hatching development in teleosts, possibly as a local paracrine and/or autocrine regulator of tissue growth (Wood et al., 2005). IM-induced morphometric changes (Table 1) in the current study were detected in characteristics related to the longitudinal axis of larvae body, therefore the IM-induced changes in MC ratios can be attributed to *igf2a* down-regulation.

In conclusion, in the current study the same experimental setting was used for analysis of gamete quality and breeding and offspring studies. Our findings were very much in line with the few available experimental results cited for humans and other mammalian vertebrates. Here, for the first time a non-mammalian vertebrate was efficiently utilized to provide new data to help clarify the adverse effects of IM on the process of reproduction. In the current study the zebrafish model enabled us to further characterize IM-induced alterations in reproductive success. This suggests that zebrafish can be considered as a suitable laboratory model not only to explain conflicting data about the effects of TKIs (such as IM) on fertility and teratogenicity, but also to uncover underlying molecular mechanisms of TKI's adverse effects reproductive processes. This follows the suggestion that zebrafish may help to establish a new path for improving fertility and treatment of ovarian disorders in vertebrates (Biswas and Maitra, 2017).

Acknowledgments

The authors are grateful to Dr. Kiranam Chatti (University of Hyderabad, India) and Dr. Kirsi Jahnukainen (University of Helsinki, Finland) for their linguistic editing and

scientific comments during preparation of this report. We gratefully acknowledge Osvah Pharmaceutical Co. (Tehran, Iran), for providing the imatinib tablet excipients.

References

- Adkins CE, Mittapalli RK, Manda VK, Nounou MI, Mohammad AS, Terrell TB, Bohn KA, YaseminC, Grothe TR, Lockman JA, Lockman PR. 2013. P-glycoprotein mediated efflux limits substrate and drug uptake in a preclinical brain metastases of breast cancer model. *Front Pharmacol* 4: 1–8.
- Ali R, Ozkalemkas F, Ozcelik T, Ozkocaman V, Ozkan A. 2006. Imatinib and Pregnancy. *Journal of Clinical Oncology*, 24(23): 3812–3813.
- Apperley J. 2009. Issues of imatinib and pregnancy outcome. *J Natl Compr Cancer Netw* 7(10): 1050–1058.
- Artac RA, McFee RM, Smith RA, Baltes-Breitwisch MM, Clopton DT, Cupp AS. 2009. Neutralization of vascular endothelial growth factor antiangiogenic isoforms is more effective than treatment with proangiogenic isoforms in stimulating vascular development and follicle progression in the perinatal rat ovary. *Biol Reprod* 81(5): 978–988.
- Arukwe A, Goksøyr A. 2003. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. *Comp Hepatol* 2(1): 4.
- Asadi-Azarbaijani B, Santos RR, Jahnukainen K, Braber S, van Duursen MB, Toppari J, Saugstad OD, Nurmio M, Oskam IC. 2017. Developmental effects of imatinib mesylate on follicle assembly and early activation of primordial follicle pool in postnatal rat ovary. *Reprod Biol* 17(1): 25–33.

642 Attar E, Balcik P, Bulgurcuoglu S, Ozsait B, Serdaroglu H, Hekim N. 2005. The role of
643 vascular endothelial growth factor (VEGF) on sperm motility and survival in
644 endometriosis. *Fertil Steril* 84: S201.

645 Ault P, Kantarjian H, O'Brien S, Faderl S, Beran M, Rios MB, Koller C, Giles F, Keating
646 M, Talpaz M, Cortes J. 2006. Pregnancy among patients with chronic myeloid
647 leukemia treated with imatinib. *J Clin Oncol* 24: 1204–1208.

648 Azanza JR, Sádaba B, Díez N (2018) Comparative Pharmacology of Tyrosine Kinase
649 Inhibitors for the Treatment of Chronic Myeloid Leukemia. *Int J Clin Pharmacol*
650 *Pharmacother* 3: 134.

651 Bahary N, Goishi K, Stuckenholtz C, Weber G, Leblanc J, Schafer CA, Berman
652 SS, Klagsbrun M, Zon LI. 2007. Duplicate VegfA genes and orthologues of the KDR
653 receptor tyrosine kinase family mediate vascular development in the zebrafish.
654 *Blood* 110(10): 3627–36.

655 Baltes-Breitwisch MM, Artac RA, Bott RC, McFee RM, Kerl JG, Clopton DT, Cupp AS.
656 2010. Neutralization of vascular endothelial growth factor antiangiogenic isoforms or
657 administration of proangiogenic isoforms stimulates vascular development in the rat
658 testis. *Reproduction* 140: 319–329.

659 Barouki, R., Gluckman, P. D., Grandjean, P., Hanson, M., Heindel, J. J. 2012. Developmental
660 origins of non-communicable disease: implications for research and public health
661 *Environ Health* 11: 1–9.

662 Barton, B.A. 2002. Stress in fishes: A diversity of responses with particular reference to
663 changes in circulating corticosteroids. *Integr Comp Biol* 42: 517–525.

664 Basciani S, De Luca G, Dolci S, et al. 2008. Platelet-derived growth factor receptor beta-
665 subtype regulates proliferation and migration of gonocytes. *Endocrinology* 149:
666 6226–6235.

667 Bay K, Bjerrum OW, Olsson-Strömberg U, Porkka K, Inge Dufva H. 2013. Reproductive
 668 hormone profiles during imatinib therapy in men with chronic myeloid leukemia.
 669 *Andrology* 2: 105.

670 Belinsky MG, Rink L, Cai KQ, Ochs MF, Eisenberg B, Huang M, von Mehren M, Godwin
 671 AK. 2008. The insulin-like growth factor system as a potential therapeutic target in
 672 gastrointestinal stromal tumors. *Cell Cycle* 7(19): 2949–55.

673 Bildik G, Akin N, Urman D, Keles I, Balaban B, Urman B, Oktem O. 2016. Imatinib
 674 mesylate accelerates follicle death and is not protective against chemotherapy induced
 675 damage in human ovary. *Fertil Steril* 106(3): e81.

676 Bott RC, McFee RM, Clopton DT, Toombs C, Cupp AS. 2006. Vascular endothelial growth
 677 factor and kinase domain region receptor are involved in both seminiferous cord
 678 formation and vascular development during testis morphogenesis in the rat. *Biol*
 679 *Reprod* 75: 56–67.

680 Brannen KC, Panzica-Kelly JM, Danberry TL, Augustine-Rauch KA. 2010. Development of
 681 a zebrafish embryo teratogenicity assay and quantitative prediction model. *Birth*
 682 *Defects Res B Dev Reprod Toxicol* 89: 66–77.

683 Braunbeck T, Lammer E. 2006. Fish embryo toxicity assays. *Aquatic Ecology & Toxicology*
 684 Department of Zoology University of Heidelberg. Im Neuenheimer Feld 230 D-69120
 685 Heidelberg Germany.

686 Challa, A.K., and Chatti, K. 2013. Conservation and early expression of zebrafish tyrosine
 687 kinases support the utility of zebrafish as a model for tyrosine kinase biology.
 688 *Zebrafish* 10(3): 264–74.

689 Christopoulos C, Dimakopoulou V, Rotas E. 2008. Primary ovarian insufficiency associated
 690 with imatinib therapy. *N Engl J Med* 358: 1079–80.

de Waal PP. 2009. Hormonal regulation of spermatogenesis in Zebrafish. Thesis, Department of Biology, Science Faculty, University of Utrecht, The Netherlands.

Eechoute K, Sparreboom A, Burger H, Franke RM, Schiavon G, Verweij J, Loos WJ, Wiemer EA, Mathijssen RH. 2011. Drug transporters and imatinib treatment: implications for clinical practice. *Clin Cancer Res* 17(3): 406–15.

Ferrara N. 2004. Vascular endothelial growth factor: basic science and clinical progress. *Endocrine Reviews* 25: 581–611.

Finn RN, Fyhn HJ, Henderson RJ, Evjen MS. 1996. The sequence of catabolic substrate oxidation and enthalpy balance of developing embryos and yolk-larvae of turbot, *Scophthalmus maximus* L. *Comp Biochem Physiol A* 115: 133–151.

Funke S, Flach E, Kiss I, Sándor J, Vida G, Bódis J, Ertl T. 2010. Male reproductive tract abnormalities: More common after assisted reproduction. *Early Hum Dev* 86:547–550.

Gambacorti-Passerini C, Tornaghi L, Cavagnini F, Rossi P, Pecori-Giraldi F, Mariani L, Cambiaghi N, Pogliani E, Corneo G, Gnessi L. 2003. Gynaecomastia in men with chronic myeloid leukaemia after imatinib. *Lancet* 361(9373):1954–6.

George S, Xia T, Rallo R, Zhao Y, Ji Z, Lin S, Wang X, Zhang H, France B, Schoenfeld D, Damoiseaux R, Liu R, Lin S, Bradley KA, Cohen Y, Nel AE. 2011. Use of a high-throughput screening approach coupled with in vivo zebrafish embryo screening to develop hazard ranking for engineered nanomaterials. *ACS Nano* 5(3): 1805–1817.

Geva E, Jaffe RB. 2000. Role of vascular endothelial growth factor in ovarian physiology and pathology. *Fertil Steril*. 74: 429–438.

Gluckman, P.D., Hanson, M.A., Cooper, C., Thornburg, K.L. 2008. Effect of in utero and early-life conditions on adult health and disease. *N. Engl. J. Med.* 359, 61–73.

Goldsmith P, Solari R. 2003. The role of zebrafish in drug discovery. *Drug Discov World* Spring.

716 Grush J, Noakes DLG, Moccia RD. 2004. The efficacy of clove oil as an anesthetic for the
717 zebrafish, *Danio rerio* (Hamilton). *Zebrafish* 1(1): 46–53.

718 Guide for the Care and Use of Laboratory Animals. 2011. National Research Council (US)
719 Committee for the Update of the Guide for the Care and Use of Laboratory Animals.
720 8th edition. Washington (DC): National Academies Press (US).

721 Gupta GS. 2005. Proteomics of spermatogenesis. Springer, Boston, MA ISBN 978-0-387-
722 27655-7.

723 Gupta T, Mullins MC. 2010. Dissection of organs from the adult zebrafish. *J Vis Exp* 37:
724 1717.

725 Hagedorn HH, Kunkel JG. 1979. Vitellogenin and vitellin in insects. *A Rev Entomol* 24:
726 475–505.

727 Hagedorn M, Carter VL. 2011. Zebrafish reproduction: revisiting in vitro fertilization to
728 increase sperm cryopreservation success. *PLoS ONE* 6(6): e21059.

729 Haigh JJ. 2008. Role of VEGF in organogenesis. *Organogenesis* 4(4): 247–56.

730 Heinrich MC, Griffith DJ, Druker BJ, Wait CL, Ott KA, Zigler AJ. 2000. Inhibition of c-kit
731 receptor tyrosine kinase activity by STI 571, a selective tyrosine kinase inhibitor.
732 *Blood* 96: 925–932.

733 Hensley ML, Ford JM. 2003. Imatinib treatment: Specific issues related to safety, fertility,
734 and pregnancy. *Semin Hematol* 40: 21–25.

735 Herr F, Liang OD, Herrero J, Lang U, Preissner KT, Han VK, Zygmunt M. 2003. Possible
736 angiogenic roles of insulin-like growth factor II and its receptors in uterine vascular
737 adaptation to pregnancy. *J Clin Endocrinol Metab* 88(10): 4811–7.

738 Hoch RV, Soriano P. 2003 Roles of PDGF in animal development. *Development* 130:4769–
739 4784.

740 Holdhoff M, Supko JG, Gallia GL, Hann CL, Bonekamp D, Ye X, Cao B, Olivi A, Grossman
741 SA. 2010. Intratumoral concentrations of imatinib after oral administration in patients
742 with glioblastoma multiforme. *J Neurooncol* 97(2): 241–5.

743 Hoo JY, Kumari Y, Shaikh MF, Hue SM, Goh BH. 2016. Zebrafish: A versatile animal
744 model for fertility research. *BioMed Res Int* 9732780.

745 Hu W, Lu S, McAlpine I, Jamieson JD, Lee DU, Marroquin LD, Heyen JR, Jessen BA. 2012.
746 Mechanistic investigation of imatinib-induced cardiac toxicity and the involvement of
747 c-Abl Kinase. *Toxicol Sci* 129(1): 188–199.

748 Ingermann RL, Schultz CLF, Kanuga MK, Wilson-Leedy JG. 2011. Metabolism of motile
749 zebrafish sperm. *Comp Biochem Physiol A* 158: 461–467.

750 Irwin DA. 2011. The regulation and function of the ovarian-derived insulin like growth factor
751 system in zebrafish (*Danio rerio*). MSc thesis in Integrative Biology, The University
752 of Guelph, Ontario, Canada.

753 Iyibozkurt AC, Balcik P, Bulgurcuoglu S, Arslan BK, Attar R, Attar E. 2009. Effect of
754 vascular endothelial growth factor on sperm motility and survival. *Reprod Biomed*
755 *Online* 19(6): 784–788.

756 James C, Trouette H, Marit G, Cony-Makhoul P, Mahon FX. 2003. Histological features of
757 acute hepatitis after imatinib mesylate treatment. *Leukemia* 17(5): 978–9.

758 Jirtle, R.L., Skinner, M.K. 2007. Environmental epigenomics and disease susceptibility. *Nat.*
759 *Rev. Genet.* 8, 253–262.

760 Kaczmarek MM, Schams D, Ziecik AJ. 2005. Role of vascular endothelial growth factor in
761 ovarian physiology—an overview. *Reprod Biol* 5: 111–136.

762 Karaman MW, Herrgard S, Treiber DK, Gallant P, Atteridge CE, Campbell BT, Chan
763 KW, Ciceri P, Davis MI, Edeen PT, Faraoni R, Floyd M, Hunt JP, Lockhart
764 DJ, Milanov ZV, Morrison MJ, Pallares G, Patel HK, Pritchard S, Wodicka

765 LM, Zarrinkar PP. 2008. A quantitative analysis of kinase inhibitor selectivity. *Nat*
 766 *Biotechnol* 126: 127–132.

767 Kent ML, Spitsbergen JM, Matthews JM, Fournie JW, Westerfield M. Diseases of zebrafish
 768 in research facilities. Zebrafish International Resource Center, Eugene, OR, 2002.
 769 https://zebrafish.org/wiki/health/disease_manual/start (accessed on 11-10-2018).

770 Kerr JB, Hutt KJ, Cook M, Speed TP, Strasser A, Findlay JK, Scott CL. 2012. Cisplatin-
 771 induced primordial follicle oocyte killing and loss of fertility are not prevented by
 772 imatinib. *Nat Med* 18: 1170–1172.

773 Kim SH, Schmitt CE, Woolls MJ, Holland MB, Kim JD, Jin SW. 2013. Vascular endothelial
 774 growth factor signaling regulates the segregation of artery and vein via ERK activity
 775 during vascular development. *Biochem Biophys Res Commun* 430(4): 1212–6.

776 King Heiden T, Carvan MJ III, Hutz RJ. 2006. Inhibition of follicular development,
 777 vitellogenesis, and serum 17 β -estradiol concentrations in zebrafish following chronic,
 778 sublethal dietary exposure to 2,3,7,8-Tetrachlorodibenzo-p-Dioxin. *Toxicol Sci* 90(2):
 779 490–499.

780 Kinkel, M.D., Eames, S.C., Philipson, L.H., Prince, V.E. 2010. Intraperitoneal injection into
 781 adult zebrafish. *J. Vis. Exp.* (42), e2126. Samaee SM, Manteghi N, Yokel
 782 RA, Mohajeri-Tehrani MR. 2018. Morphometric characteristics and time to hatch as
 783 efficacious indicators for potential nanotoxicity assay in zebrafish. *Environ Toxicol*
 784 *Chem* 37(12): 3063–3076.

785 Korpelainen E, Karkkainen M, Tenhunen A, Lakso M, Rauvala H, Vierula M, Parvinen M,
 786 Alitalo K. 1998. Overexpression of VEGF in testis and epididymis causes infertility in
 787 transgenic mice: evidence for nonendothelial targets for VEGF. *J Cell Biol* 143:
 788 1705–1712.

789 Legros L, Bourcier C, Jacquel A, Mahon FX, Cassuto JP, Auberger P, Pagès G. 2004.
790 Imatinib mesylate (STI571) decreases the vascular endothelial growth factor plasma
791 concentration in patients with chronic myeloid leukemia. *Blood* 104(2):495–501.

792 Li J, Chu L, Sun X, Liu Y, Cheng CH. 2015. IGFs mediate the action of LH on oocyte
793 maturation in zebrafish. *Mol Endocrinol* 29(3): 373–383.

794 Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time
795 quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Method* 25:402–408.

796 Lodish MB, Stratakis CA. 2010. Endocrine side effects of broad-acting kinase inhibitors.
797 *Endocr Relat Cancer* 17: R233–R244.

798 Lodish MB. 2013. Kinase Inhibitors: Adverse effects eelated to the endocrine system. *J Clin*
799 *Endocrinol Metab* 98(4): 1333–1342.

800 Loren AW, Mangu PB, Beck LN, Brennan L, Magdalinski AJ, Partridge AH, Quinn
801 G, Wallace WH, Oktay K, American Society of Clinical Oncology. 2013. Fertility
802 Preservation for Patients With Cancer: American Society of Clinical Oncology
803 Clinical Practice Guideline Update. *J Clin Oncol* 31(19): 2500–2510.

804 Maley D, Laird AS, Rinkwitz S, Becker TS. 2013. A simple and efficient protocol for the
805 treatment of zebrafish colonies infected with parasitic nematodes. *Zebrafish* 10(3):
806 447–50.

807 Malozowski S, Nelson L, Calis KA. 2008. More on ovarian insufficiency with imatinib. *N*
808 *Engl J Med* 358: 2648–2649.

809 Mariani S, Basciani S, Fabbri A et al. 2011. Severe oligozoospermia in a young man with
810 chronic myeloid leukemia on long-term treatment with imatinib started before
811 puberty. *Fertil Steril* 95: 1120.e15– e17.

812 Mariani S, Giona F, Basciani S, Brama M, GnessiLow L. 2008. Bone density and decreased
813 inhibin-B/FSH ratio in a boy treated with imatinib during puberty. *Lancet* 372: 111–
814 112.

815 Matthews JL. 2004. Common diseases of laboratory zebrafish. *Methods Cell Biol* 77:617–
816 643.

817 Mauduit C, Hamamah S, Benahmed M. 1999 Stem cell factor/c-kit system in
818 spermatogenesis. *Hum Reprod Update* 5:535–545.

819 McFee RM, Artac RA, McFee RM, Clopton DT, Smith RA, Rozell TG, Cupp AS. 2009.
820 Inhibition of vascular endothelial growth factor receptor signal transduction blocks
821 follicle progression but does not necessarily disrupt vascular development in perinatal
822 rat ovaries. *Biol Reprod* 81: 966–977.

823 Miranda AC, Bazzoli N, Rizzo E, Sato Y. 1999. Ovarian follicle atresia in two teleost
824 species: a histological and ultrastructural study. *Tissue Cell* 31(5):480–488.

825 Mughal TI, Schrieber A. 2010. Principal long-term adverse effects of imatinib in patients
826 with chronic myeloid leukemia in chronic phase. *Biologics: Targets & Therapy* 4:
827 315–323.

828 Nakayama Y, Yamamoto T, Abé SI. 1999. IGF-I, IGF-II and insulin promote differentiation
829 of spermatogonia to primary spermatocytes in organ culture of newt testes. *Int J Dev*
830 *Biol* 43(4): 343–7.

831 Nalbandian A, Dettin L, Dym M, Ravindranath N. 2003. Expression of vascular endothelial
832 growth factor receptors during male germ cell differentiation in the mouse. *Biol*
833 *Reprod* 69(3):985–94.

834 Nelson SN, Van Der Kraak G. 2010. The role of the insulin-like growth factor (IGF) system
835 in zebrafish (*Danio rerio*) ovarian development. *Gen Comp Endocrinol* 168: 103–110.

836 Nóbrega RH, Morais RD, Crespo D, de Waal PP, de França LR, Schulz RW, Bogerd J. 2015.
837 Fsh stimulates spermatogonial proliferation and differentiation in zebrafish via *Igf3*.
838 Endocrinology 156(10): 3804–17.

839 Novak M, Žegura B, Nunić J, Gajski G, Gerić M, Garaj-Vrhovac V, Filipič M. 2017.
840 Assessment of the genotoxicity of the tyrosine kinase inhibitor imatinib mesylate in
841 cultured fish and human cells. Mutat Res. 814: 14–21.

842 Novartis Pharmaceuticals Corporation. Gleevec® Highlights of Prescribing Information.
843 Revised 7/2018. East Hanover, New Jersey. Available at:
844 http://www.pharma.us.novartis.com/product/pi/pdf/gleevec_tabs.pdf (accessed on 24-
845 01-2019).

846 Nurmio M, Toppari J, Zaman F, et al. 2007 Inhibition of tyrosine kinases PDGFR and C-Kit
847 by imatinib mesylate interferes with postnatal testicular development in the rat. Int J
848 Androl 30:366–376.

849 Obermair A, Obruca A, Pöhl M, Kaider A, Vales A, Leodolter S, Wojta J, Feichtinger W.
850 1999. Vascular endothelial growth factor and its receptors in male fertility. Fertil
851 Steril 72: 269–275.

852 Ohkubo N, Matsubara T. 2002. Sequential utilization of free amino acids, yolk proteins and
853 lipids in developing eggs and yolk-sac larvae of barfin flounder *Verasper moseri*. Mar
854 Biol 140:187–196.

855 Ohyashiki K, Kuriyama Y, Nakajima A, Tauchi T, Ito Y, Miyazawa H, Kimura Y, Serizawa
856 H, Ebihara Y. 2002. Imatinib mesylate-induced hepato-toxicity in chronic myeloid
857 leukemia demonstrated focal necrosis resembling acute viral hepatitis. Leukemia
858 16(10): 2160–1.

859 Patel BB, He YA, Li X.-M, Frolov A, Vanderveer L, Slater C, Schilder RJ, Mehren MV,
 860 Godwin AK, Yeung AT. 2008. Molecular mechanisms of action of imatinib mesylate
 861 in human ovarian cancer: a proteomic analysis. *Cancer Genom Proteom* 5: 137–150.
 862 Peng B, Lloyd P, Schran H. 2005. Clinical pharmacokinetics of imatinib. *Clin*
 863 *Pharmacokinet* 44(9): 879–94.
 864 Phillips HS, Hains J, Leung DW, Ferrara N. 1990. Vascular endothelial growth factor is
 865 expressed in rat corpus luteum. *Endocrinology* 127: 965–967.
 866 Pink D, Schoeler D, Lindner T, Thuss-Patience PC, Kretzschmar A, Knipp H, Vanhoefer
 867 U, Reichardt P. 2005. Severe hypoglycemia caused by paraneoplastic production of
 868 IGF-II in patients with advanced gastrointestinal stromal tumors: a report of two cases.
 869 *J Clin Oncol* 23: 6809–6811.
 870 Prabhash K, Sastry PSRK, Biswas G, Bakshi A, Prasad N, Menon H, Parikh PM. 2005.
 871 Pregnancy outcome of two patients treated with imatinib. *Ann Oncol Annals of*
 872 *Oncology* 16(12): 1983–1984.
 873 Pye SM, Cortes J, Ault P, Hatfield A, Kantarjian H, Pilot R, et al. 2008 The effects of
 874 imatinib on pregnancy outcome. *Blood* 111:5505–8.
 875 Raimondi C, Fantin A, Lampropoulou A, Denti L, Chikh A, Ruhrberg C. 2014. Imatinib
 876 inhibits VEGF-independent angiogenesis by targeting neuropilin 1-dependent ABL1
 877 activation in endothelial cells. *J Exp Med* 211(6): 1167–83.
 878 Ravindranath N, Little-Ihrig L, Phillips HS, Ferrara N, Zeleznik AJ. 1992. Vascular
 879 endothelial growth factor messenger ribonucleic acid expression in the primate ovary.
 880 *Endocrinology* 131: 254–260.
 881 Rossteuscher S, Schmidt-Posthaus H, Schäfers C, Teigeler M, Segner1 H. 2008. Background
 882 pathology of the ovary in a laboratory population of zebrafish. *Dis Aquat Org* 79:169–
 883 172.

884 Safian D, Morais RD, Bogerd J, Schulz RW. 2016. Igf binding proteins protect
885 undifferentiated spermatogonia in the zebrafish testis against excessive
886 differentiation. *Endocrinology* 157(11): 4423–4433.

887 Samaee S.-M, Manteghi N, Yokel RA, Mohajeri-Tehrani MR. 2018. Morphometric
888 characteristics and time to hatch as efficacious indicators for potential nanotoxicity
889 assay in zebrafish. *Environ Toxicol Chem* 37(12): 3063–3076.

890 Samaee SM, Patzner RA, Mansour N. 2009. Morphological differentiation within the
891 population of Siah Mahi, *Capoeta capoeta gracilis*, (Cyprinidae, Teleostei) in a river
892 of the south Caspian Sea basin: a pilot study. *J Appl Ichthyol* 25: 583–590.

893 Samaee SM. 2015. Experimental assessment of the efficacy of five veterinary broad-
894 spectrum anthelmintics to control the intestinal capillariasis in zebrafish (*Danio*
895 *rerio*). *Zebrafish*. 12(3): 255–67.

896 Samaee, S.-M., Rabbani, S., Jovanović, B., Mohajeri-Tehrani, M.R., Haghpanah, V. 2015.
897 Efficacy of the hatching event in assessing the embryo toxicity of the nano-sized TiO₂
898 particles in zebrafish: A comparison between two different classes of hatching-
899 derived variables. *Ecotox. Environ. Safe.* 116, 121–128.

900 Schultheis B, Nijmeijer BA, Yin H, Gosden RG, Melo JV. 2012. Imatinib mesylate at
901 therapeutic doses has no impact on folliculogenesis or spermatogenesis in a leukaemic
902 mouse model. *Leuk Res* 36: 271–274.

903 Selman K, Wallace RA, Sarka A, Qi X. 1993. Stages of oocyte development in the zebrafish,
904 *Brachydanio rerio*. *J Morphol* 218: 203–224.

905 Seshadri T, Seymour JF, McArthur GA. 2004. Oligospermia in a patient receiving imatinib
906 therapy for the hypereosinophilic syndrome. *N Engl J Med* 351:2134–2135.

907 Silva P, Rocha MJ, Cruzeiro C, Malhão F, Reis B, Urbatzka R, Monteiro RAF, Rocha E.
908 2012. Testing the effects of ethinylestradiol and of an environmentally relevant

909 mixture of xenoestrogens as found in the Douro River (Portugal) on the maturation of
 910 fish gonads-A stereological study using the zebrafish (*Danio rerio*) as model. *Aquat*
 911 *Toxicol* 124: 1–10.

912 Soriano P. 1994 Abnormal kidney development and hematological disorders in PDGF beta-
 913 receptor mutant mice. *Genes Dev* 8:1888–1896.

914 Soriano P. 1997 The PDGF alpha receptor is required for neural crest cell development and
 915 for normal patterning of the somites. *Development* 124:2691–2700.

916 Subhasri B, Sudipta M. 2017. Let's talk and grow together: a bidirectional communication
 917 between granulosa- and oocyte-derived factors in the ovary. *Glob J Reprod Med* 1(4):
 918 555569.

919 Teng M, Qi S, Zhu W, Wang Y, Wang D, Yang Y, Li H, Li C, Dong K, Wang C. 2017. Sex-
 920 specific effects of difenoconazole on the growth hormone endocrine axis in adult
 921 zebrafish (*Danio rerio*). *Ecotoxicol Environ Saf* 144: 402–408.

922 Tybulewicz VL, Crawford CE, Jackson PK, et al. Neonatal lethality and lymphopenia in mice
 923 with a homozygous disruption of the c-abl proto-oncogene. *Cell* 1991;65:1153–1163.

924 Tyler CR, Sumpter JP. Oocyte growth and development in teleosts. 1996. *Rev Fish Biol Fish*
 925 6: 287–318.

926 Van Stappen G. 1996. Artemia. In: Manual on the production and use of live food for
 927 aquaculture. Eds. Lavens, P., Sorgeloos, P. FAO Fish Tech Pap. No. 361. FAO, Rome.
 928 p. 39-130.

929 Varga ZM. 2011. Aquaculture and husbandry at the Zebrafish international resource center.
 930 *Method Cell Biol* 104.

931 Wallace RA. 1985. Vitellogenesis and oocyte growth in non-mammalian vertebrates. In:
 932 Browder LW, ed, *Developmental Biology*, Plenum Press, New York, 1:127–177.

933 Wang J, Zhu X, Zhang X, Zhao Z, Liu H, George R, Wilson-Rawls J, Chang Y, Chen Y.
 934 2011. Disruption of zebrafish (*Danio rerio*) reproduction upon chronic exposure to
 935 TiO₂ nanoparticles. Chemosphere 83: 461–467.

936 Weber LP, Hill RL Jr, Janz DM. 2003. Developmental estrogenic exposure in zebrafish
 937 (*Danio rerio*): II. Histological evaluation of gametogenesis and organ toxicity. Aquat
 938 Toxicol 63: 431–446.

939 Westerfield M. 2000. The Zebrafish book: A Guide for the Laboratory Use of Zebrafish
 940 (*Danio rerio*). University of Oregon Press, Eugene, USA.

941 Westerfield M. 2007. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish
 942 (*Danio rerio*), 5th Edition. University of Oregon Press, Eugene.

943 Winnicki A, Radziun MS, Radziun K. 1970. Structural and mechanical changes in the egg
 944 membranes of *Salmo gairdneri* Rich. during the period of hatching of the larvae. *Acta*
 945 *Ichth Piscat* 1: 7–18.

946 Wirbisky SE, Weber GJ, Sepúlveda MS, Lin T.-L, Jannasch AS, Freeman JL. 2016. An
 947 embryonic atrazine exposure results in reproductive dysfunction in adult zebrafish and
 948 morphological alterations in their offspring. Sci Rep6: 21337.

949 Wolff NC¹, Richardson JA, Egorin M, Ilaria RL Jr. 2003. The CNS is a sanctuary for
 950 leukemic cells in mice receiving imatinib mesylate for Bcr/Abl-induced leukemia.
 951 *Blood* 101(12): 5010–3.

952 Wood AW, Duan C, Bern HA. 2005. Insulin-like growth factor signaling in fish. Int Rev
 953 Cytol 243: 215–85.

954 Yaghmaei P, Parivar K, Gharibi A, Nabiuni M. 2009a. The biological effects of imatinib on
 955 male fertility of wistar rats. Int J Fertil Steril 3(3): 135–142.

956 Yaghmaei P, Parivar K, Jalalvand F. 2009b. Effect of imatinib on the oogenesis and pituitary-
 957 ovary hormonal axis in female wistar Rat Int J Fertil Steril 3(1): 11–16.

958 Yamamoto, M., 1975. Hatching gland and hatching enzyme. In: Yamamoto T, ed, *Medaka*
959 *(Killifish): Biology and Strains*. Keigaku Pub Co, Tokyo, pp 73–79.

960 Yang H, Jones C, Varga ZM, Tiersch TR. 2007. Development of a simplified and
961 standardized protocol with potential for high-throughput for sperm cryopreservation
962 in zebrafish *Danio rerio*. *Theriogenology* 68(2): 128–136.

963 Yildiz C, Kacan T, Akkar OB, Karakus S, Seker M, Kacan SB, Ozer H, Cetin A. 2016. Effect
964 of imatinib on growth of experimental endometriosis in rats. *Eur J Obstet Gynecol*
965 *Reprod Biol* 197: 159–63.

966 Zamah AM, Mauro MJ, Druker BJ, Oktay K, Egorin MJ, Cedars MI, Rosen MP. 2011. Will
967 imatinib compromise reproductive capacity? *The Oncologist* 16: 1422–1427.

968 Zhang Y, Qiao H, Chen C, Wang Z, Xia X. 2016. Determination of nitrofurans metabolites
969 residues in aquatic products by ultra-performance liquid chromatography-tandem
970 mass spectrometry. *Food Chem* 192: 612-7.

971 Zhu Y, Ma X, Su G, Yu L, Letcher RJ, Hou J, Yu H, Giesy JP, Liu C. 2015. Environmentally
972 Relevant Concentrations of the flame retardant tris (1,3-dichloro-2-propyl) phosphate
973 inhibit growth of female zebrafish and decrease fecundity. *Environ Sci Technol*
974 49(24): 14579–87.

975

976

977

978

979

980

981

982

Tables

Table 1. IM-induced changes in larval morphometric ratios.

Footnote → Values superscripted with the same letter are not significantly different. See the legend of Figure 1 for abbreviations of the morphometric ratios. Number of specimens (n) = 20.

Table 2. Regression equations, correlation (r^2), F and p values of the significant relationships found between the larval morphometric ratios and the frequency of exposure of adult zebrafish to IM.

Footnote → The boldface and underlined data show morphometric ratios that are correlated to the frequency of exposure to IM by simple regression models in the form of a linear and quadratic function, respectively. See the legend of Figure 1 for abbreviations of the morphometric ratios.

Figures

Fig. 1. Observation time points, whole-body zebrafish sagittal section, breeding trials, and morphometric characteristics. (a) Observation time points of zebrafish embryos and larvae. (b) Whole-body zebrafish, paraffin embedded, and H & E stained. Sagittal orientation, dorsal up. S, swim bladder; O, ovary; L, liver; and I, intestine. (c-d) Breeding trials (IM-0♀ × IM-0♂ [c] and IM-3♀ × IM-3♂ [d]). (e) Male replacement trial (IM-3♀ × IM-0♂). (f) Female replacement trial (IM-0♀ × IM-3♂). (g-j) Morphometric characteristics determined in zebrafish embryos and larvae. The landmarks drawn on the schematics of the zebrafish larvae at 48, 72, 96, and 120 hpf depict the characteristics utilized for screening IM-induced responses. BL (total body length): greatest horizontal body distance — anterior-most part of head to the end of body. APB (anterior part of the body): anterior-most part of head to the posterior-most insertion of yolk sac. PoPB (posterior part of body): the posterior-most insertion of yolk sac to the end of body. HL (head length): anterior-most part of head to the place where the head is connected to the body. BD1 (body depth 1): vertical distance from posterior-most insertion of yolk sac to upper surface of body. BD2 (body depth 2): greatest vertical body distance. Fifteen ratios were calculated from the six morphometric characteristics BL/APB, BL/PoPB, BL/BD1, BL/BD2, BL/HL, APB/PoPB, APB/BD1, APB/BD2, APB/HL, PoPB/BD1, PoPB/BD2, PoPB/HL, BD1/BD2, BD1/HL, and BD2/HL.

Fig. 2. Follicles at different gametogenic stages (a-h) and atretic follicles (i-l). (a) Perinucleolus stage (PN); (b,c) early cortical alveolus stage (eCA); (d,e) mid-cortical alveolus stage (mCA); (f-g) vitellogenic stage (V); (h) mature stage (M), and (i-l) examples of atretic oocytes at mature stage.

Fig. 3. Folliculogenesis in untreated (control) and IM-treated fish. Percentage (a, b, c, d, and e) and diameter (μm ; f, g, h, i, and j) of ovarian follicles at various gametogenic stages. See the legend of Figure 2 for the abbreviation of follicular stages. Results that do not share the same letter are significantly different. $n = 32$.

Fig. 4. Percentage (a, b, c, d, and e) and diameter (f, g, h, i, and j) of follicles at various stages in relationship to the frequency of IM exposure. See the legend of Figure 2 for the abbreviation of follicular stages. $n = 32$.

Fig. 5. Folliculogenesis (percentage [a, b, c, d, and e] and diameter [f, g, h, i, and j] of follicles) at termination of IM exposure (black bars) and after the 1-month depuration period (IMD) (gray bars). See the legend of Figure 2 for the abbreviation of follicular stages. Results that do not share the same letter are significantly different. $n = 48$. IM: the sum of IM-1, IM-2 and IM-3, IMD: the sum of IM-1, IM-2 and IM-3 after the one month depuration.

Fig. 6. Sperm quality parameters in untreated (control) and IM-treated fish. Density, per mL, ($n = 35$) (a) and motility ($n = 20$) at different “seconds post activation”, spa of sperm. Results that do not share the same letter are significantly different.

Fig. 7. Density, per mL, ($n = 35$) and motility ($n = 20$) of sperm in relationship with the frequency of exposure to IM.

Table. 1. IM-induced changes in larval morphometric ratios.

No.	Variables	IM exposure frequency			
		0	1	2	3
1	BL/BD1-72	9.33 ± 0.09 ^b	8.80 ± 0.04 ^a	8.75 ± 0.15 ^a	9.68 ± 0.10 ^c
2	APB/BD1-72	3.61 ± 0.06 ^b	3.52 ± 0.16 ^{bc}	3.33 ± 0.11 ^a	3.74 ± 0.11 ^c
3	BL/BD1-96	10.23 ± 0.09 ^c	9.71 ± 0.11 ^b	9.60 ± 0.22 ^b	8.96 ± 0.03 ^a
4	APB/BD1-96	3.96 ± 0.05 ^c	3.70 ± 0.14 ^b	3.56 ± 0.07 ^a	3.53 ± 0.07 ^a
5	APB/BD2-96	2.53 ± 0.06 ^c	2.36 ± 0.07 ^b	2.32 ± 0.05 ^{ab}	2.25 ± 0.01 ^a
6	BL/BD1-120	11.70 ± 0.58 ^d	10.13 ± 0.22 ^a	10.04 ± 0.14 ^a	10.83 ± 0.15 ^b
7	BL/APB-120	2.67 ± 0.05 ^{bc}	2.63 ± 0.08 ^{ab}	2.56 ± 0.05 ^a	2.72 ± 0.04 ^c
8	BL/BD2-120	7.16 ± 0.24 ^d	6.18 ± 0.45 ^a	6.44 ± 0.11 ^{ab}	6.68 ± 0.10 ^b
9	BL/HL-120	3.77 ± 0.11 ^a	3.72 ± 0.05 ^a	3.81 ± 0.11 ^a	4.03 ± 0.05 ^b
10	PoPB/BD2-120	4.48 ± 0.13 ^b	3.84 ± 0.34 ^a	3.92 ± 0.16 ^a	4.24 ± 0.03 ^b
11	BD2/HL-120	0.53 ± 0.02 ^a	0.60 ± 0.04 ^b	0.59 ± 0.01 ^b	0.59 ± 0.02 ^b
12	BL/BD1-144	11.65 ± 0.15 ^c	10.29 ± 0.47 ^a	10.61 ± 0.17 ^a	11.02 ± 0.11 ^b
13	BL/BD2-144	7.02 ± 0.25 ^b	6.58 ± 0.28 ^a	6.92 ± 0.09 ^b	7.08 ± 0.07 ^b
14	PoPB/BD1-144	7.47 ± 0.08 ^c	6.55 ± 0.14 ^a	6.87 ± 0.38 ^{ab}	6.97 ± 0.18 ^b
15	BD1/BD2-144	0.60 ± 0.01 ^a	0.64 ± 0.01 ^b	0.65 ± 0.02 ^b	0.64 ± 0.02 ^b
16	BD1/HL-144	0.32 ± 0.02 ^a	0.36 ± 0.02 ^b	0.35 ± 0.02 ^b	0.35 ± 0.01 ^{ab}
17	BL/HL-168	3.74 ± 0.10 ^a	4.03 ± 0.04 ^c	3.87 ± 0.03 ^b	3.69 ± 0.07 ^a
18	BL/BD1-168	10.59 ± 0.67 ^a	11.32 ± 0.08 ^b	11.47 ± 0.12 ^b	11.27 ± 0.07 ^b
19	APB/HL-168	1.36 ± 0.09 ^{ab}	1.47 ± 0.08 ^b	1.44 ± 0.06 ^{ab}	1.35 ± 0.04 ^a
20	BL/BD1-192	12.10 ± 0.33 ^c	11.05 ± 0.31 ^a	11.61 ± 0.11 ^b	12.06 ± 0.06 ^c
21	APB/BD1-192	4.34 ± 0.08 ^b	4.07 ± 0.04 ^a	4.00 ± 0.04 ^a	4.36 ± 0.14 ^b
22	APB/BD2-192	2.57 ± 0.05 ^b	2.47 ± 0.08 ^a	2.40 ± 0.04 ^a	2.69 ± 0.05 ^b
23	BL/BD1-216	12.64 ± 0.40 ^b	11.75 ± 0.22 ^a	11.47 ± 0.29 ^a	12.60 ± 0.13 ^b
24	BL/HL-240	3.76 ± 0.06 ^a	3.78 ± 0.04 ^a	4.06 ± 0.01 ^b	4.08 ± 0.01 ^b
25	BL/BD1-240	11.48 ± 0.21 ^a	11.76 ± 0.79 ^{ab}	12.22 ± 0.29 ^b	13.29 ± 0.15 ^c
26	PoPB/BD1-240	7.14 ± 0.13 ^a	7.59 ± 0.43 ^b	7.83 ± 0.04 ^b	8.30 ± 0.14 ^c

Values superscripted with the same letter are not significantly different. n = 5 (for each treatment). See the legend of Figure 1 for abbreviations of the morphometric ratios.

Table 2. IM exposure frequency effect on larval morphometric ratios. Regression equations, correlation (r^2), and F values of the significant relationships found between the larval morphometric ratios and the frequency of exposure of adult zebrafish to IM-0, IM-1, IM-2, and IM-3.

No.	Variables Independent	Equation	r^2	F
<u>1</u>	<u>BL/BD1-72</u>	<u>$y = 0.365x^2 - 0.995x + 9.354$</u>	<u>0.933</u>	<u>90.440</u>
2	IPB/BD1-72	$y = 0.018x + 3.522$	0.013	0.183
3	BL/BD1-96	$y = -0.392x + 10.213$	0.881	103.998
4	IPB/BD1-96	$y = -0.144x + 3.901$	0.742	40.186
5	IPB/BD2-96	$y = -0.088x + 2.497$	0.758	43.787
6	BL/BD1-120	$y = 0.591x^2 - 2.046x + 11.672$	0.839	33.842
7	BL/IPB-120	$y = 0.010x + 2.630$	0.021	0.299
8	BL/BD2-120	$y = 0.306x^2 - 1.038x + 7.097$	0.609	10.132
9	BL/HL-120	$y = 0.090x + 3.696$	0.491	13.490
10	PoPB/BD2-120	$y = 0.241x^2 - 0.788x + 4.453$	0.665	12.912
<u>11</u>	<u>BD2/HL-120</u>	<u>$y = -0.020x^2 + 0.077x + 0.532$</u>	<u>0.600</u>	<u>9.750</u>
<u>12</u>	<u>BL/BD1-144</u>	<u>$y = 0.443x^2 - 1.484x + 11.568$</u>	<u>0.726</u>	<u>17.263</u>
13	BL/BD2-144	$y = 0.051x + 6.820$	0.050	0.743
14	PoPB/BD1-144	$y = -0.116x + 7.139$	0.114	1.809
<u>15</u>	<u>BD1/BD2-144</u>	<u>$y = -0.012x^2 + 0.049x + 0.603$</u>	<u>0.620</u>	<u>10.600</u>
16	BD1/HL-144	$y = 0.007x + 0.335$	0.120	1.901
17	BL/HL-168	$y = -0.117x^2 + 0.321x + 3.757$	0.723	17.006
18	BL/BD1-168	$y = 0.217x + 10.835$	0.290	5.707
19	IPB/HL-168	$y = -0.008x + 1.415$	0.015	0.213
<u>20</u>	<u>BL/BD1-192</u>	<u>$y = 0.376x^2 - 1.081x + 12.013$</u>	<u>0.650</u>	<u>12.098</u>
<u>21</u>	<u>IPB/BD1-192</u>	<u>$y = 0.157x^2 - 0.471x + 4.348$</u>	<u>0.799</u>	<u>25.857</u>
<u>22</u>	<u>IPB/BD2-192</u>	<u>$y = 0.097x^2 - 0.263x + 2.581$</u>	<u>0.074</u>	<u>18.429</u>
<u>23</u>	<u>BL/BD1-216</u>	<u>$y = 0.505x^2 - 1.555x + 12.677$</u>	<u>0.798</u>	<u>25.745</u>
24	BL/HL-240	$y = 0.122x + 3.738$	0.813	61.022
25	BL/BD1-240	$y = 0.587x + 11.309$	0.699	32.584
26	PoPB/BD1-240	$y = 0.371x + 7.159$	0.798	55.337

See figure 1 legend for morphometric ratio abbreviations. Number of specimens = 20.

(a) **(b)** **(c)**

99.5
0.0
90.3
89.5

← FR →
← HR-48 →
← HR-72 (total hatching) →
← SR-24-240 →

(d)

77.9
55.2
88.2
88.2

(e)

88.6
22.5
95.8
95.8

← FR →
← HR-48 →
← HR-72 (total hatching) →
← SR-24-240 →

(f)

88.8
0.0
99.2
99.2

(g) **(h)** **(i)** **(j)**

(a) **(b)** **(c)** **(d)** **(e)** **(f)** **(g)**

(a) **(b)** **(c)** **(d)** **(e)** **(f)** **(g)**