Review on the use of zebrafish embryos to study the effects of anesthetics during early development

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ABSTRACT

Over the years, the potential toxicity of anesthetics has raised serious concerns about its safe use during pregnancy. As evidence emerged from research in animal models, showing that some anesthetic drugs are potential teratogenic, the determination of the risk of exposures to anesthetic drugs at early life stages became mandatory. However, due to inaccessibility and ethical constrains related to experimental conditions, the use of early life stages in mammalian models is limited. In this regard, some animal and nonanimal models have been suggested to surpass mammalian use in experimentation. Among them, the zebrafish embryo test has been recognized as a promising alternative in toxicology research, as well as an inexpensive and practical test. Substantial information collected from developmental research following compounds exposure, has contributed to the application of zebrafish assays in research, although only a few studies have focused on the use of early life stages of zebrafish to evaluate the developmental effects of anesthetics. Based on the recent advances of science and technology, there is a clear potential for zebrafish early life stages to provide new insights into anesthetics teratogenicity. This review provides an overview of recent anesthesia research using zebrafish embryos, demonstrating its usefulness to the anesthesia field, discussing the recent findings on various aspects related to the effects of anesthetics during early life development and the strengths and limitations of this model system.

INTRODUCTION

Little is known about the long-term impacts of many anesthetic agents during pregnancy and their outcomes to the fetus, which is of concern; it is estimated that around 0.5–2% of pregnant women require nonobstetric surgery involving anesthesia (Allaert et al. 2007; Reitman and Flood 2011). In
recent years, the understanding of the physiology and cellular biology of anesthesia on the developing brain has emerged from different studies in various species (Cottrell and Hartung 2012; Andropoulos 2018). These have originated a debate about the safety of anesthetics drugs and their adverse consequences to the central nervous system (CNS) development (Sun 2010; Hudson and Hemmings 2011; Vutskits and Davidson 2017; Culley and Avram 2018), thus, drawing the attention of international organizations (Zhou and Ma 2014; Andropoulos and Greene 2017). In this sense, many efforts have been made over the years, but questions remain unanswered concerning the pharmacotoxicity of anesthetic drugs during early development. Indeed, until now, the clinical data available relative to these drugs in early developmental stages are inconclusive and based only on epidemiological studies (Sanders et al. 2013; Kang et al. 2017; Vutskits and Davidson 2017).

Furthermore, critical periods such as organogenesis (from the 3rd to the 8th week of gestation in humans) are not accessible for analysis in nonhuman primate embryos, thereby limiting embryological studies (Ostrer et al. 2006). In addition to the technical difficulties, the use of animals rise ethical issues (Festing and Wilkinson 2007). Therefore, the use of animal models and in vitro cellular or tissue preparations in embryological research is critical to study the toxicological and mechanistic responses of anesthetics (Schumann 2010; Saint-Raymond and de Vries 2016). However, animal ethics is a very important issue that has been a subject of heated debate for many years (Festing and Wilkinson 2007). This is particularly interesting and challenging considering studies using early developmental stages as critical period varies from species to species (Dobbing and Sands 1979). On the other hand, in vitro methods have been established for the assessment of anesthetics toxicity in early developmental stages (Olsen and Brambrink 2013) and, although in vitro studies remove some of the ethical issues concerning human and other animals’ embryo studies, they are not equivalent to whole-animal experiments. In response to this dilemma, the principle of Replacement, part of the 3Rs (Replacement, Reduction, and Refinement) principle to the use of animals for scientific purposes has been implemented over the years (Schechtman 2002).

Replacement consists of the proposal of several alternatives to animal models to overcome ethical issues, as the use of in vitro models or early life stages of zebrafish; this last approach, as also uses animals, is often called a relative replacement (Redmond 2014). In vitro models help to study biological responses and mechanisms related to human health and disease, but they are not equivalent to whole-animal experiments. Therefore, early life stages of zebrafish have become an alternative to mammalian testing in toxicology and biomedical research, especially due to genetic, developmental, and brain similarities (Doke and Dhawale 2015). These similarities raise some concern particularly when zebrafish is used with the claim that experiments cause less harm in this animal model. However, zebrafish can only be used as replacement up to 5 days postfertilization (dpf), i.e. when the yolk reserves are still present and before it can feed independently (EU 2010). From the legal point of view, prior to yolk depletion, the embryos and larvae do not account as protected animals (Strahle et al. 2012). It is considered that when the animal starts to feed independently, it also develops behaviors and mechanisms to chase preys and escape from predators, being this moment an approximate cue to the activity of relevant neuroanatomical features thought to be consistent with the capacity to experience pain or suffering. Thus, in safety pharmacological investigations and toxicology studies, zebrafish models are in a strategic place to predict toxicity of drugs since these experiments are complex, expensive, and time consuming when higher vertebrate models are used, and in vitro models are too simple (Figure 1). In fact, some of the earliest works to understand the fundamentals of general anesthetics and their mechanism of action were performed in this teleost fish. Therefore, this review describes the current knowledge on the toxicological effects associated with the use of anesthetics in the early life stages of the lower
vertebrate zebrafish and reviews evidence of the use of this animal model to provide insights into the molecular basis of anesthesia.

Figure 1: The zebrafish model as a tool to anesthesia toxicity screening. Anesthesia research may benefit from the zebrafish as an alternative to other animal models, overcoming practical and/or ethical reasons from experiments with higher vertebrates. Zebrafish embryos and larvae may be used after in vitro (e.g. cell lines) studies and before mammalian animal models. The developmental process of zebrafish is highly comparable to that of mammals allowing an alternative and fast route for screening and identification of chemicals before preclinical evaluation in mammalian models.

**2. An overview on the importance of the zebrafish model**

Over the last decade, the number of publications listed in PubMed with zebrafish has duplicated (from 1548 in 2007 to 3503 in 2018), in part due to its applicability to study processes with relevance to human health (Barros et al. 2008). In fact, the zebrafish embryo has gained an important role for pediatric research (Ali et al. 2011). There are several advantages in using this model comparative to other animal models (Giacomotto and Segalat 2010), which have been extensively reviewed elsewhere (Veldman and Lin 2008; Ali et al. 2011; Vliegenthart et al. 2014). Briefly, this model has a low cost, small size, high fecundity, genetic similarity (around 70%) with humans and rapid growth (embryos hatch within 72 h postfertilization (hpf)—Figure 2—and reach sexual maturity at approximately 3–4 months), thus, allowing feasible longitudinal studies (Soares et al. 2009; Coimbra et al. 2015). Moreover, zebrafish embryogenesis is non-placental, external to the mother, and all stages of development are visually accessible under light microscope during the first days of development (Kimmel et al. 1995; Westerfield 2000; Spence et al. 2008; Truong et al. 2011; Strahle et al. 2012). Indeed, the transparency of zebrafish embryos allows the application of visualization techniques that, with intra-uterine embryos, would be difficult or uneconomical. Furthermore, the similar neurodevelopment of the zebrafish embryo in comparison with other vertebrate models (Appel 2000; Lewis and Eisen 2003; Saint-Amant 2006; Schmidt et al. 2013; Semple et al. 2013; Oosterhof et al. 2015), whose hallmarks are presented in Figure 3, supports its application as a complementary tool to conventional models to fill the gap between simple in vitro cell-based assays
and more complex whole animal systems for toxicity screening in early stages (Ali et al. 2011). In fact, the effects of anesthetics in the development can be detected very early, as the first spontaneous movements are detected at 17 hpf (Saint-Amant and Drapeau 1998); the progenitors of tissues and organs are formed at 36 hpf (Spence et al. 2008; Taylor et al. 2010) and active avoidance behaviors at 120 hpf (Spence et al. 2008). Despite the advances in the knowledge of these processes and in the zebrafish embryonic development, the understanding of the developmental effects of anesthetics drugs is still limited. In this sense, studies have been conducted using zebrafish early life stages to explore anesthetics’ effects on development and their potential toxicity mechanisms.

Figure 2. Translating times across human, rat, and zebrafish early development. The human embryo and rodent development are very similar with only slight differences between species. In both cases, up to 144 hpf (hours postfertilization), only a cluster of cells that will originate the blastocyst are observed. By the contrary, the embryonic development of zebrafish is very rapid and morphologically different with all major organs formed in the first 24 hpf and within 72 hpf, the fish hatch and start swimming. Human embryo images via Wikimedia Commons; rodent images adapted from Veeck and Zaninovic (2003) and O3; Zebrafish images were adapted from “Go Fish!” (2009) poster from Genesi. h: hours.

3. Developmental toxicity of anesthetic drugs in zebrafish

Despite zebrafish brain being simpler than the rodents and mammalian brain, its formation, differentiation, and connectivity of neurons require molecular mechanisms that appear to be conserved in all vertebrates (Tropepe and Sive 2003). Similarly the counterparts of mammalian brain subdivisions are morphologically identifiable in zebrafish larva (Nishimura et al. 2015). A comparative neuroanatomy at the time at which neurons differentiate into mature neurons is present in Figure 4. Although the telencephalon is evverted and not evaginated like in mammals, the lateral pallium of teleost’s appears to be structurally homologous to the mammalian hippocampus, instead of the medial pallium from which derive in humans (Rodriguez et al. 2002). Understanding these
differences and particularities will increase the translational potential of the research results. Still, zebrafish embryonic development is much faster; the nervous system is formed at 1 dpf and by 3 dpf, the blood—brain barrier is observed (Jeong et al. 2008).

Figure 3: Hallmarks of neurodevelopment during zebrafish development and the corresponding embryonic day (E) or gestational week (GW) in rats and humans, respectively. The formation of neural progenitors during early stages accompanied by an increase in cell proliferation are the first steps in the induction of the neuroectoderm which gives rise to the central and peripheral nervous systems, culminating in the generation of mature neurons. Key neurodevelopmental processes where anesthetics may interfere are presented in bold. Numbers between parenthesis represent hours postfertilization.

The major neurotransmitter systems in zebrafish are detected between 20 and 24 hpf (Higashijima et al. 2004; Cox et al. 2005; Panula et al. 2010; Rico et al. 2011; Horzmann and Freeman 2016) and, at 5 dpf, the larvae already present a maturated neurotransmitter system and a broad range of behaviors (Schneider 2017). In humans, neurotransmitters may be found as soon as the egg is fertilized but the major neurotransmitter systems appear around the 13th gestational week while in rats they appear around embryonic day 16 (Herlenius and Lagercrantz 2001; Ritter et al. 2001; Semple et al. 2013). However, despite their functionalities, their quantity change during pre and postnatal development (Herlenius and Lagercrantz 2001). Anesthetics acts at the CNS mainly through mechanisms that involve voltage-gated channels and neurotransmitter systems. However, the anesthetic teratogenicity may be independent of the main mechanism of action of the anesthetics (Felix et al. 2014, 2018). Nevertheless, several studies showed the influence of anesthetics in the modulation of neurodevelopmental processes, which may result in neurotoxic damage in the immature brain and in long-term behavioral effects (Gohlke et al. 2008; Rand-Weaver et al. 2013).
fact, the developing brain is particularly vulnerable to anesthetics exposure during neurodevelopmental processes such as neurogenesis, synaptogenesis, and immature glial development (Lei et al. 2012). In order to promote developmental effects, an anesthetic drug must be administered in a dose sufficient to induce a particular defect in a fetus/embryo during a critical development point (Upadya and Saneesh 2016). The findings concerning the use of anesthetic drugs in early zebrafish developmental stages are summarized in Table 1 and described below. A selection of studies regarding the effects of some main anesthetics in early phase of development of zebrafish, and potential implications in clinical practice are showed. It is worth noting that in early zebrafish developmental stages, substances are mainly tested by immersion in a bath at the desired concentration, but microinjection can also be an alternative method of administration (Schubert et al. 2014). The use of an immersion bath of anesthetics is similar to the use of volatile agents in mammals, as, in both cases, the intake of the anesthetics is by inhalation. When zebrafish larvae are used, this uptake is through rudimentary gills (depending of the larval stage) and/or through the skin (Martins et al. 2018). During the embryo stages, the anesthetic has to enter into the chorion before reaching the embryo, and so the concentrations in the embryos have to be measured to understand which concentration is really causing an impact (Felix et al. 2016). Despite of this, the concentrations used in zebrafish might not be so distant from those found in humans’ plasma (Robinson et al. 2016).

![Figure 4: Brain comparative neuroanatomy across zebrafish, rat, and human at the time the major neurotransmitter systems are detected. In zebrafish, major neurotransmitters may be found as soon as 48 hpf (hours postfertilization). In comparison, major neurotransmitters are found in rats around embryonic day 16 (E16) and in humans around gestational week 13 (GW13). Ce: cerebellum (Ce and MO are part of the hindbrain); F: forebrain; M: midbrain; MO: medulla oblongata; OB: olfactory bulb; Sc: spinal cord. 3.1: Ketamine

Ketamine is the most studied anesthetic in zebrafish early development. Ketamine is a noncompetitive N-methyl-d-aspartate (NMDA) receptor antagonist (Sleigh et al. 2014) which induces sedation, hypnosis, and some analgesia. In human medical settings, its dissociation effects are noticed at concentrations around 0.2–0.4 nM, while hypnosis is obtained with concentrations 20—higher, around 8 mM (Sleigh et al. 2014). In humans, ketamine can be found at a higher concentration in fetal blood than in maternal blood, and it has been advised for use in late pregnancy in low concentrations (Walton and Melachuri 2006; Neuman and Koren 2013). However, the potential implications to the fetus of ketamine exposure are not fully understood. Previous research studies have highlighted the effects of ketamine in zebrafish early developmental stages. The first study exploring ketamine effects in the early life stages of the zebrafish dates back to 2007 when Burgess and Granato treated zebrafish larvae with 0.1–3.0 mM ketamine for 20 min and observed altered...
sensorimotor gating (Burgess and Granato 2007). Using a similar approach, exposure to a ketamine concentration of 0.5 mM induced changes in startle habituation and startle sensitivity (Wolman et al. 2011). More recently, a dose-dependent effect on the heart rate of 26 and 52 hpf dechorionated zebrafish embryos and changes in mitogen-activated protein kinase (MAPK) signaling pathway after exposure to 0.5–10 mM of ketamine for 2 or 20 h has been shown (Kanungo et al. 2012). Also, ketamine has been shown to affect spinal motor neurons after 20 h of exposure to concentrations of 0.5–2.0 mM (Cuevas et al. 2013). The previously described CNS defects induced by ketamine exposure were further corroborated and changes in the expression pattern of genes involved in motor neuron development were detected (Kanungo et al. 2013). The acute administration of ketamine at concentrations ranging from 0.84 to 3.37 mM for 20 min during the blastula period caused teratogenic effects producing a variety of abnormal malformations (Felix et al. 2014). The exposure at later developmental stages (gastrula and segmentation) resulted in a decrease of the malformations observed suggesting an embryonic stage-dependent teratogenicity (Felix et al. 2016). The observed teratogenic effects were associated with an oxidative imbalance that could be the key factor for ketamine toxicity (Felix et al. 2016). Furthermore, using the same methodological approach, ketamine has been shown to induce behavioral alterations (Felix et al. 2017) and apoptosis-related gene expression changes (Felix et al. 2017). It is therefore possible that, the observed teratogenic effect may be a consequence of the association of oxidative stress-related impairment and apoptosis during ketamine exposure (Felix et al. 2016). Similar malformations, behavioral dysfunctions and p53-dependent apoptosis were observed when exposing embryos for 24 h during early development at concentrations ranging from 0.21 to 0.38 mM (Felix et al. 2017, 2018), further validating the teratogenic potential of ketamine to this species. After a 24 h exposure to 2 mM ketamine, 72 hpf embryos showed alterations on cyp19 expression levels and on MAPK pathway, associated with the reduction of estradiol-17β levels that is very important to neurodevelopment and neuroprotection (Trickler et al. 2014). More recently, 52 hpf embryos were treated for 24 h with 0.1–2.0 mM ketamine, and the anesthetic dose (2 mM) induced a reduction in the serotonergic areas of the brain, and an inhibition of endogenous serotonin metabolism to 5-hydroxyindole acetic acid (5-HIAA) (Robinson et al. 2015). A later work also reported a dose-dependent decrease in dopamine levels and an inhibition of tyrosine hydroxylase and dopamine metabolizing enzyme gene expression changes after applying the same methodology (Robinson et al. 2016). Lately, it has shown the occurrence of developmental toxicity at 56 hpf larvae when exposed for 72 h at similar concentrations (2 mM). At later stages, 72 hpf, exposure to ketamine for 24 h induced a dose-dependent overexpression of cyp3a65, a specific drug metabolizing CYP enzyme (Robinson et al. 2017). When treatment occurred at early stages such as gastrula (around 6 hpf), an overall retardation of the embryo development was observed (Robinson et al. 2017) as described earlier (Felix et al. 2016). In addition, exposure to the same concentration (2 mM) affects bioenergetics and gene expression pathways in the zebrafish embryos reducing ATP levels, mitochondrial membrane potential and the total mitochondrial proteins (Robinson et al. 2018). More recently, a 2-h exposure of 28 hpf embryos to ketamine concentrations closer to the lower values of the human anesthetic range (2 mM) was shown to cause significant effects in the heart rate; furthermore, when exposed for 20 h, the embryos presented a decreased size. Additionally, this longer exposure induced neuron toxicity by decreasing the area occupied by serotonergic neurons in the brain and by reducing tyrosine hydroxylase-immunoreactive neurons (Robinson et al. 2018). In general, current studies have shown that ketamine exposure during early developmental phases is, at a certain degree, harmful to zebrafish development and have helped to validate zebrafish embryos as a rational animal model for the study of the developmental effects of ketamine. Generally, effects in zebrafish were detected when exposure is done as early as 3–52 hpf (corresponding from cleavage to 1–2 months of gestation in humans), using ketamine concentrations
lower (Felix et al. 2014, 2016) or equivalent to human anesthetic plasma concentration (2 mM) (Robinson et al. 2016). However, the molecular mechanism underlying ketamine toxicity in early developmental phases remains elusive and further studies are required; this will be important to establish strategies to ensure a normal development.

3.2: Propofol

Propofol is a short-acting, gamma-aminobutyric acid (GABA) receptor agonist; it is also a hypnotic agent widely used in pediatric procedures due to its rapid induction and fast recovery (Key et al. 2010). It is worth noting that under medical situations the mean target propofol concentration required to produce a satisfactory sedation in human is approximately 11 mM (Blayney et al. 2003). Propofol has been considered safe to use during pregnancy, however, there is still a debate regarding subtle associations (Reitman and Flood 2011). Similarly to ketamine, several studies have reported that propofol induced widespread apoptosis and behavioral deficits in the developing rodent models (Creeley et al. 2013; Yu et al. 2013). Yet, little is known about its effects on the cellular and molecular mechanisms during the sensitive early life stages. A recent published work showed that zebrafish embryos exposed to propofol from 6 to 48 hpf at concentrations as low as 1 mM presented aberrant development phenotypes (pericardial cysts, an unconsumed yolk sac, small eyes, bleeding, and a curly spinal cord) and an increase in apoptosis through changes in apoptotic-related gene expression. Moreover, propofol induced the inhibition of myelination basic protein expression, thus, supporting the teratogenic potential of this drug (Guo et al. 2015; Liu et al. 2018). In addition, a dose-dependent loss-of-neural activity was also observed after larvae exposure to propofol concentrations higher than 30 mM (Du et al. 2018). However, additional experiments are required to explore the molecular mechanism underlying the propofol neurotoxic effects in this species.

3.3: Etomidate

Etomidate is an intravenous anesthetic that potentiates the GABA neurotransmitter system by altering the transmembrane chloride conductance (Tobias 2015). This compound is an interesting alternative to the most common intravenous anesthetics because etomidate possesses some advantages that make it an attractive option to use in pediatric procedures (Mandt et al. 2012) although its neuronal and cognitive effects remain elusive particularly during early infancy (Nyman et al. 2016). In human neonates and infants clinical settings, the minimal hypnotic plasma concentration is around 1 mM (Su et al. 2015) and no teratogenic effects have been described in the literature for its administration during pregnancy in clinically effective doses. However, there is a lack of adequate and well-controlled studies during gestation and early development for etomidate (Perna et al. 2015). In this regard, a recent study reported that general anesthesia of 6 dpf larval zebrafish has been achieved at concentrations of 30 mM, causing the loss of brain functions (Du et al. 2018). Still, further studies are necessary to conclude on the teratogenic potential of this anesthetic.

3.4: Benzodiazepines

Benzodiazepines are GABA receptor agonists acting in the CNS to induce sedation, muscle relaxation, some degree of amnesia and anticonvulsant activity (Griffin et al. 2013). Among them,
Midazolam is a sedative-hypnotic agent also used in pediatric medicine, and it has been shown to accumulate in zebrafish larvae (7 dpf) after a 3-h exposure period to concentrations of 1 mM (Alderton et al. 2010). This represents a lower concentration relative to the mean maximum plasma concentration (4.47 mM) following intravenous administration in children (Malinovsky et al. 1993). A similar benzodiazepine, diazepam, administered to zebrafish embryos for 3 days, induced alterations on locomotion (1 nM) and on the genes involved in the circadian rhythm (both at 1 nM and 1 mM) (Oggier et al. 2010). In addition, 6 dpf zebrafish exposed to higher concentrations (up to 2.5 mM) of diazepam had motor function impairments, but no alterations on anxiety-like responses (Zahid et al. 2018). As the binding site for benzodiazepines in the brain is described to be highly conserved between fish and mammals (Carr and Chambers 2001), and as there are contradictory data regarding the use of these drugs during pregnancy (Neuman and Koren 2013), zebrafish seems to be a good model to clarify this issue, evaluating possible implications to humans and other animals.

3.5: Volatile agents

Inhalation of volatile anesthetics has been the preferred technique in obstetrics and pediatrics. The administration of isoflurane has been associated with accelerated apoptosis and teratological effects in mammals (mice, rats, rhesus macaque, and humans) in concentrations ranging from 32.5 to around 146 mM (Brambrink et al. 2010; Istaphanous et al. 2011; Davidson 2016). The information regarding the effect of these agents in zebrafish larvae is scarce; still, an effective dose of isoflurane for anesthetic purposes of 14.2 mM isoflurane has been established for 7 days postfertilization larvae while a 10 higher concentration has been defined as the mean lethal dose for this species (Ku et al. 2013). More studies with isoflurane and other halogenated anesthetics are important to further assess the potential adverse effects and correspondent toxicity mechanisms of these compounds in early life forms using zebrafish pharmacological assays.

3.6: Local anesthetics

Local anesthetics are usually amino amides that act through blockade of the sodium channels of the neuron membrane, interfering with the action potential and thus, preventing propagation of this signal along the axon (Cox et al. 2003). In this sense, the use of local anesthetics might affect indirect cell mitosis and embryogenesis. However, until now, no firm evidence exists that any local anesthetic agent is teratogenic in humans or in rodent models (Ramazzotto et al. 1985; Fujinaga and Mazze 1986; Fleming and Alderton 2013). One example of a local anesthetic used both in mammals and fish is lidocaine. It is usually administered by immersion in fish, and can be used as an analgesic and/or anesthetic. Most studies revealed no teratogenic effects caused by lidocaine at concentrations higher than the ones that are clinically relevant (Fujinaga 1998), but, in an in vitro mouse study, this drug caused neural closure defects (O’Shea and Kaufman 1980). More recently, lidocaine concentrations superior to 20 mg/L (around 74 mM, and higher than clinical relevant concentrations) have been shown to induce teratogenic effects on zebrafish embryos (2 hpf) exposed for 48 h (Lomba et al. 2019). Still, there are contradictory results between studies with different rat strains (Teiling et al. 1987; Smith et al. 1989), which may reveal an effect dependent on species and/or strain. Thus, some groups of people may be susceptible to lidocaine teratogenic effects, which needs further genetic evaluation. As zebrafish is a simpler model than rodents, with high genetic homology with humans, this seems to be the adequate animal model to shed light on this issue.
3.7: Opioids

These agents include morphine-like agonists, meperidine-like agonists such as meperidine and fentanyl and synthetic opioid analogs (Babb et al. 2010). High doses of opioids are capable of producing anesthesia in humans (Bovill et al. 1984; Vilela et al. 2009), and they are frequently used for pain management during pregnancy, despite evidences of possible adverse effects on fetal development, such as poor fetal growth, preterm birth, birth defects and neonatal abstinence syndrome (Yazdy et al. 2015). However, observational studies in humans are always limited and more research needs to be done. Up until now, no evidence supported the teratogenicity of these compounds in animal models (Fujinaga et al. 1986; Fujinaga and Mazze 1988; Fujinaga et al. 1988). Still, behavioral outcomes following opioids exposure have been linked with changes in brain gene expression (Przewlocki 2004) which have also been described in zebrafish embryos (Herrero-Turrion et al. 2014; Sanchez-Simon et al. 2010). However, some compounds seem to be less harmful in zebrafish than in rodents, such as morphine (Ali et al. 2011) which increased mortality in rodents (Creeley et al. 2013). In zebrafish, opioid effects have been recently reviewed (Demin et al. 2018; Bao et al. 2019). Overall, morphine enhanced cell proliferation, as already seen in mammals, but neuronal differentiation seems to be dependent on the zebrafish embryos age (Sanchez-Simon et al. 2010). Another study revealed the effect of chronic morphine exposure for 19 h in 5 hpf embryos, where it altered the expression of genes associated with neuronal development, such as CNS patterning processes, differentiation, and neurotransmission (dopaminergic, serotonergic, and glutamatergic) and the activation of axonogenesis and dendritogenesis (Herrero-Turrion et al. 2014). At concentrations lower than those clinically relevant (higher than 245 mM) (Aguirre et al. 2016), chronic exposure of zebrafish embryos to morphine disrupts the immune responses, similar to what was observed in humans (Mottaz et al. 2017). More recently, behavioral alterations were observed when exposing 120 hpf larvae to concentrations around 3.5 mM (1 mg/L) for 40 min (Lopez-Luna et al. 2017) and also when 168 hpf were exposed for 1 h at 10 higher concentrations (Zhou et al. 2018). In addition, when 5 hpf embryos were exposed for 19 h to morphine concentrations of 10 nM novel evidence has emerged showing a delay in neural stem cells differentiation and changes in the expression of some transcription factors (Jimenez-Gonzalez et al. 2018). Therefore, morphine may pose a risk to early pregnancy, which is worthy of further research.

4: DISCUSSION

Through the years, concerns have been raised regarding early embryo–fetal development during the prenatal period. Indeed, this period could represent many risks to the developing fetus and to the mother, especially in the beginning when pregnancy symptoms are often unrealized and anesthetics’ safety for this condition is not taken into consideration (Goetzl 2010). Moreover, it is estimated that about 2% of first trimester pregnant women undergo general anesthesia, which might increase the risk of prenatal adverse effects (Allaert et al. 2007; Reitman and Flood 2011). Despite the observed developmental toxicity of anesthetics in animal models, the application of these findings and its translation to humans is still uncertain. The introduction of zebrafish in standard laboratory practices has allowed the investigation of genetic, cellular, morphological, and biochemical mechanisms by which anesthetics induce developmental deficits. Overall, and although no clear mechanism has been described for the developmental effects induced by anesthetics, the references presented here
and others in the literature present the zebrafish early developmental stages as a robust and complementary promising model to better understand the mechanism of action of these compounds during the neurodevelopmental period (Kanungo et al. 2014; Guo et al. 2015; Felix et al. 2016, 2017; Nishimura et al. 2016; d’Amora and Giordani, 2018). So far, alterations in morphology, behavior, oxidative stress, enzymes, and genetic expression related with apoptosis, proliferation, and other neuronal processes were reported. These findings appear to indicate similar multicellular processes affected by anesthetics in zebrafish development, as well as in rodents and humans. To some extent, the cross-translation is possible due to the highly conserved mechanisms of anesthetics’ action in vertebrates (Stewart et al. 2011), indicating that some developmental effects of anesthetics can be similar between zebrafish and higher vertebrates. Also, zebrafish brain morphology, neurochemistry, and neuroendocrine system are homologous to those recognized in mammals (Kalueff et al. 2014). While the mechanisms of action underlying anesthesiainduced embryo–fetal developmental toxicity remain elusive, this review highlights the potential for further studies that can be conducted to examine changes induced by anesthetic compounds in zebrafish. However, there are also some obstacles to this model application to anesthesia research that should be taken into consideration. For instance, despite sharing with mammals a similar, but old diverged ancestor (Woods et al. 2000), zebrafish lacks some organs, such as lungs, limbs, and others (Ali et al. 2011), which prevent the evaluation of anesthetics’ effects in these organs. Though, zebrafish has been used to study lung disease by studying neutrophilic inflammation that is responsible for much of the tissue damage seen in many lung diseases (Renshaw et al. 2007; Ljujic et al. 2018). Other difference compared to other vertebrates, is the whole-genome duplication in zebrafish (Woods et al. 2000) which can significantly complicate genetic approaches as gene subfunctionalization and neofunctionalization occurred (Postlethwait et al. 2004). Moreover, from a physiological perspective, zebrafish and humans have very different respiratory systems due to the transition from water to land, leading to the development of a double circulation and progression from ectothermic-heterothermy to endothermic-homeothermy. However, the embryological origin of the fish gills and of the mammalian lungs is the same, the primitive pharynx (Carvalho and Gonçalves 2011). As zebrafish is a poikilothermic model, it can have different metabolic and clinical responses (induction and recovery times) to the anesthetics (Neiffer and Stamper 2009). In fact, it has already been documented significant variations in oxygen consumption during developmental stages (Bang, Gronkjaer, and Malte 2004; Stackley et al. 2012; Huang et al. 2013). Moreover, the ability to control depth and duration of anesthesia, as well as monitor physiological parameters during early development in this species is a crucial and difficult issue. The control of the anesthesia depth in humans involves technology that does not exist for this small teleost fish. However, even in humans there is no completely/consensual accepted methodology that accurately detects and monitors the depth of general anesthesia (Ferreira et al. 2019) and clinical responses have not been replaced since antiquity. Fish anesthetic delivery is also mainly determined by assessing clinical responses such as activity, reactivity to stimuli, equilibrium, muscle tone, and respiratory and heart rates. Interestingly, fish show the same response as other species when volatile anesthetics are used. Induction usually takes 5–10 min and is marked by a decrease in swimming, respiratory rate, and reaction to stimuli, followed by loss of the equilibrium. The surgical anesthesia stage is observed when the previous measures are further depressed and there is a loss of reaction to painful stimuli. Recovery from anesthesia follows a similar pattern to the mammals’ recovery (Sneddon 2012). Additionally, important considerations for modeling anesthesia in zebrafish related to the complexity of brain development and function need to be clarified. In this regard, and despite comparative brain neuroanatomy, which has revealed similar structural properties to higher vertebrates (Kalueff et al. 2014), experimental conditions in zebrafish are still difficult to extrapolate to humans due to differences in the proliferation rates during...
neurodevelopmental period of zebrafish (Schmidt et al. 2013; Nishimura et al. 2015), which may induce different vulnerabilities and susceptibilities to anesthetics. Moreover, there is a gap in the knowledge of certain brain functions, as well as in the importance of some CNS structures and their mammalian counterparts, thus, complicating the interpretation of physiological states involving the modulation of a variety of neural circuits. The efficacy and route of administration during early developmental stages are other challenges, as the presence of the chorion up to 48 hpf may hinder the passage of some anesthetics, resulting in different pharmacokinetics. Yet, this can be overcome by a manual or automated chorion-removing process (Bugel et al. 2014) or by drug microinjection. Moreover, drugs which are not water-soluble can be problematic to administer by water immersion and the use of solvents can impact biotransformation of compounds, which is a critical process for the toxic outcome (David et al. 2012). Moreover, the knowledge regarding the characterization of functional metabolic capacities expressed in zebrafish embryos is scarce and pivotal for the correct interpretation of teratogenicity assays (Verbueken et al. 2017). Overall, even considering these limitations, at long-term, zebrafish early life stages may constitute a highly applicable model in advancing anesthesia research with high potential and translational value.

5: CONCLUSION

This review highlighted the biological advances in anesthesia research that have been facilitated by the use of early developmental stages of zebrafish, providing new and exciting possibilities to understand the prenatal effects of the anesthetics. Through the studies summarized here, some areas and anesthetics were identified as needing more research to clarify potential teratogenic effects and increase the knowledge on brain development and function under anesthesia in early developmental stages. Although some biological problems remain to be addressed with this model, zebrafish will allow the use of a complex organism instead of in vitro techniques and replaces the use of animal models more prone to pain, potentiating knowledge that can be more directly applied to other vertebrates, thereby, facilitating the translational biomedical research and reducing the risks associated in clinical practice.

Declaration of interest

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Table 1: Experimental studies carried out over the last years on the developmental effects of anaesthetic exposure in the zebrafish during early developmental stages.

<table>
<thead>
<tr>
<th>Anaesthetic</th>
<th>End Point</th>
<th>Zebrafish stage</th>
<th>Concentration tested (mM)</th>
<th>Exposure duration (min)</th>
<th>Key observations</th>
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<td>Significant reduction in ERK/MAPK activity</td>
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<td>Reduce of motor neuron axon length and decrease both cranial and spinal motor neurons</td>
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<td>Pharyngula (28 hpf)</td>
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<td>Concentration-dependent increase in anomalies and mortality</td>
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<td>0.84-3.37</td>
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<td>Stage-dependent bone and cartilage malformations</td>
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<td>Blastula (2.5 hpf)</td>
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<td>Pharyngula (28 hpf)</td>
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<td>Altered ATP synthase gene expression</td>
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## Oxidative stress indicators

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<th>Time (h)</th>
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<td>Dose-related increase in GSH levels</td>
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<td>Hatching (52 hpf)</td>
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<td>Dose-dependent modulation of 5-HT and 5-HIAA levels</td>
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<tr>
<td>Hatching (52 hpf)</td>
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<td>Dose-dependent modulation of 5-HT and 5-HIAA levels</td>
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<td>Hatching (48 hpf)</td>
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## Neurotransmitter metabolic activity

<table>
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<tr>
<th>Stages</th>
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<th>Time (h)</th>
<th>Effect</th>
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<td>1200</td>
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<td>(Robinson et al., 2018)</td>
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<tr>
<td>Segmentation (10.5 hpf)</td>
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<td>Altered expression of genes related to serotonergic neurons</td>
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<tr>
<td>Hatching (52 hpf)</td>
<td>0.1-2.0</td>
<td>1444</td>
<td>Reduced serotonergic activity and altered expression of genes related to tyrosine hydroxylase and dopamine metabolizing enzyme</td>
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</tr>
<tr>
<td>Hatching (48 hpf)</td>
<td>2.0</td>
<td>1200</td>
<td>Reduced serotonergic activity and altered expression of genes related to tyrosine hydroxylase and dopamine metabolizing enzyme</td>
<td>(Robinson et al., 2016)</td>
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## Propofol

<table>
<thead>
<tr>
<th>Effect</th>
<th>Gastrula (6 hpf)</th>
<th>Time (h)</th>
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<td>Developmental phenotyping</td>
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<tr>
<td>Gene expression</td>
<td>0.001-0.009</td>
<td>2520</td>
<td>Dose-dependent decrease in hatchability</td>
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<tr>
<td>Behavioural phenotyping</td>
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<td>Increased aberrations</td>
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<tr>
<td>Pharmacokinetics</td>
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<td>Increased apoptosis</td>
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<tr>
<td>Larval (6 dpf)</td>
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<td>10 to 120</td>
<td>Dose-dependent loss of brain function</td>
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<tr>
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<td>0.003 to 0.1</td>
<td>10 to 120</td>
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<td>Midazolam</td>
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<td>Significant accumulation in animals</td>
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<td>Larval (7 dpf)</td>
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<td>Gene expression</td>
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<td>Blastula (5 hpf)</td>
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**Abbreviations:** hpf: hours post-fertilization; dpf: days post-fertilization