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Zebrafish (*Danio rerio*): Ethics in animal experimentation

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Abstract: *Animal experimentation has had great importance in the development of biomedical research; it has been responsible for major medical advances in recent decades. Even with the great progress of alternative methods, the animal model is still needed. The zebrafish (Danio rerio) is an animal model that has reached high popularity for its features, mainly because it has some similarities to mammals, which bestow it a great potential as an experimental model. Although fish have lower scientific classification when compared to mammals, the concern with the ethical aspects for using the former in research should be taken into account by including them in welfare assessment, pain perception and nociception surveys, and methods of anesthesia and euthanasia. The review discusses the concept of welfare, anesthesia, and euthanasia, specifically for the zebrafish.*

Keywords: *animal experimentation, ethics, zebrafish*

I. Introduction

The use of animals in biomedical research allows multiple scientific and technological advances that have influenced the development of vaccines, antibiotics, and anesthetics [1]. Animal research has contributed 70% of the Nobel Prizes in Physiology or Medicine, and award-winning scientists say they could not do their research without animals [2].

Although some models have been replaced by alternative methods that meet the in vivo tests, scientific research still needs animal models for development, reliability, and legitimacy of science. Of course, nowadays, experiments in humans are unethical; reports of abuses in research are staggering and prowl the research, such as reports from the time of World War These amazing cases resulted in codes and laws, for example, the Nuremberg Code (1947), which determined that experimental results with animals were used as the basis for humans, and the Declaration of Helsinki (1964), which has already recognized the care that should be taken in conducting experiments that could affect the environment and the welfare of animals [3].

When *Bioethics: Bridge to the Future* was published, in 1971, its author, Van Rensselaer Potter, the creator of bioethics, opened the way for a new ethical science that combines humility, responsibility, and respect for life. Ethics, a set of knowledge extracted from the investigation of human behavior in a rational, based, scientific, and theoretical way, had another important factor to be noted: the weighting of actions, mainly taking another lives into account; the welfare of those animals that help support science.

The approval of the *Arouca Law* [4] is an important step to regulate the use of animals for experimentation in Brazil, even though this law is centered on the power of the Ministry of Science and Technology, which weakens the approach to animal protection [5].

With the increasing use of zebrafish in animal experimentation, due to its genetic homology [6] and also cellular homology with mammals [7], this species has also called attention to ethics regarding its experimentation, therefore, increasing the number of questions about the welfare and methods of anesthesia and euthanasia in this species.

II. Welfare

In 1985, in the United States, there was a review process of the North-American law of animal welfare [8] after the controversial case of *Silver Spring* (in a search, monkeys were kept in terrible sanitary and physical conditions) and made mandatory the review of research protocols in animals by institutional commissions [9]. In Brazil, the National Commission on Ethics in Research and the Research Ethics Committees were formed, they are in Resolution No. 196 of 1996 of the Brazilian National Health Council.

In order to guide the ethics committees in research, the doctrine of the three "Rs" (refinement, replacement, and reduction) was established in 1959 in England. Refinement is the use of methods that alleviate or minimize potential pain, suffering, or distress, and improve welfare for animals that need to be used by

trained personnel. Replacement is biomedical research involving the use of methods such as cell cultures and computer models instead of animals. Mammals must be replaced by animals whose nervous system is less developed. Reduction presents the idea of always using the fewest animals possible, in as much as it provides significant statistical results.

According to Royal Society for the Prevention of Cruelty to Animals [10] between 1955 and 2013 the number of scientific procedures using fish reported in the Home Office annual statistics on animal use increased from 131,100 to 507,400. Zebrafish alone accounted for 330,161 of the procedures undertaken in 2013, the second most commonly used group of animals after rodents. Of the 1.93 million experimental procedures in 2014, the majority involved mice (60%), fish (14%), following by rats and birds [11].

The zebrafish has been thought as an alternative method in which mammalian animals could be replaced by animals whose nervous system is less developed and are similar to the target model. Researchers confirmed that fish are neurologically equipped with nociceptors (sensory receptors that send signals that cause pain perception) and unconscious emotional responses, but they do not suffer pain and aware feelings [12]. In the one research [13], the zebrafish is the animal model for studies of nociceptors. In another research [14] the results show that the zebrafish avoided having contact with seven types of anesthetics, including tricaine methanesulfonate (MS-222), isoeugenol, quinaldine sulfate, and benzocaine. The zebrafish did not show any aversion to etomidate neither to 2.2.2 tribromoethanol (TBE) [14]. *Danio rerio* have changed their behavior in response to the presence of alarm substances, moving on stereotypes of pain and fear situations (as in predation models) [15].

Researchers mention how difficult is distinguishing nociception and pain in fish [16]. In order to improve the interpretation of results that have been published, a comprehensive catalog of zebrafish behavior have been developed, it is the ZBC (*Zebrafish Behavior Catalog*), which covers both adult models and larvae [17]. There is a glossary in the ZBC that cites the specific behaviors, referring to them by numbers, standardizing the identification of zebrafish behavior [17].

They are animals of a lower scientific classification; the concern with the ethical aspects regarding the use of such animals in research must be taken into consideration. A study suggests that most ethics committees evaluate procedures in vertebrates, in which zebrafish is included, but, sometimes, this species can be one of the least evaluated by ethics committees [9]. As a means of minimizing pain and stress caused by experiments, methods of anesthesia and euthanasia become important to reduce these situations.

III. Use Of Anesthetics

To evaluate methods of euthanasia in animals, some criteria in anesthetics must be taken into consideration, such as the ability to induce loss of consciousness, death with the least possible pain, minimal stress or anxiety, reversibility of consciousness, and time required to induce unconsciousness, all appropriate to the type of species and the age of the animal [16]. The ideal anesthetic is one that produces anesthesia in three minutes or less and does not cause toxicity in treatment levels [18].

One of the most widely used anesthetics in zebrafish is the tricaine methanesulfonate (MS-222). This anesthetic inhibits the propagation of action potentials by blocking sodium channels of the membrane. The effectiveness of MS-222 depends on environmental factors, for example: temperature, oxygen, pH, hardness and salinity of the water, and even biological factors such as age, sex, size, weight, lipid content, species of fish, and density of biomass [19]. Studies suggest a dose of 50-400 mg/L^{-1}, or 250-500 mg/L for most species [20, 21]. There are several physiological effects of MS-222, such as changes of blood plasma, hypoxia, respiratory acidosis, and hyperglycemia [19]. The same study used buffered and unbuffered MS-222 in euthanasia and verified that the latter caused stress in 100% of zebrafish, which was evidenced by the rapid opercular movements [19]. None of the animals exposed to buffered MS-222 showed rapid opercular movements.

There are still other possible anesthetics for use in fish, such as benzocaine, metomidate, eugenol, isoeugenol, 2-phenoxyethanol, quinaldine, and quinidine sulfate. Nonsteroidal anti-inflammatory agents such as ketoprofen and carprofen can also be used.

Benzocaine or benzocaine hydrochloride is a dipping solution to be prepared at concentrations > 250 mg/L, and must be buffered [21].

Metomidate is a non-barbiturate hypnotic that activates and modulates inhibition of gamma-aminobutyric acid receptor (GABA) type A, is a sedative for human and veterinary uses. The deleterious effects on fish are reduced breathing and circulation, and subsequent hypoxia and decreased pH in the blood. The 2-phenoxyethanol is commonly used in vaccines, skin care products, and perfumes; the precise mode for using it on fish is not known, but may involve the membrane of neuronal cells [20].

Eugenol and isoeugenol are clove oil components; they inhibit sodium, potassium, and calcium channels. Both cause depression of the cardiovascular system, reduce blood pressure, and increase blood plasma catecholamines, indicating a response to stress by the use of this anesthetic [22]. The proposed concentration depends on several factors, but should be below 17 mg/L, and high concentrations can euthanize. The animal

must be kept at least ten minutes in solution until the cessation of its opercular movements. These components are carcinogenic according to the National Toxicology Program [21], thus, they must be handled judiciously by trained personnel.

Researchers found that the zebrafish avoid contact with MS-222, but they found less evidence of this aversion in the exhibition to metomidate and to clove oil, suggesting that these agents are less aversive to the zebrafish [18]. Furthermore, those authors also suggested that exposure to MS-222 caused increased anxiety.

Eugenol was considered more efficient due to its high solubility, resulting in rapid induction, even when a low concentration is used [23]. Researchers speculated that the properties of clove oil (eugenol 95%) cover the gills, and hence, these properties prolong the effects of anesthesia [24].

Regarding costs, compared with MS-222, metomidate is six times more expensive, benzocaine is 20 times cheaper, 2-phenoxyethanol is 30 times cheaper, and clove oil is 100 times cheaper.

Some researchers concluded that metomidate and clove oil are alternative methods recommended for zebrafish euthanasia [18]. Anothers also affirmed that clove oil is a suitable agent in studies that evaluate stress because this agent does not interfere with detection of cortisol increase [25]. Nevertheless, MS-222 remains a reliable and consistent anesthetic agent [26].

IV. Euthanasia

Death must be confirmed by observing the cessation of the vital signs of the animal. Euthanasia is performed by three basic mechanisms: hypoxia, depression of neurons, and physical disruption of brain activity [21]. According to the Canadian Council of Animal Care [27] and Brazilian laws [28] euthanasia of fish must be carried out in two steps: anesthesia until loss of balance; followed by a chemical or physical method that causes brain death. According to European Union [29], the methods may be used on unconscious animals, providing the animal does not regain consciousness before death. The killing of animals shall be completed by one of the following methods: confirmation of permanent cessation of the circulation, destruction of the brain, dislocation of the neck, exsanguination or confirmation of the onset of *rigor mortis*.

Euthanasia can succeed by excessive MS-222, a common anesthetic in zebrafish euthanasia, by hypoxia [16]. In Canada, the use of lethal levels of depressants of the central nervous system, such as the buffered MS-222, is the preferred method of euthanasia [27]. In the United States, MS-222 must be buffered, the concentration depends on the species, large fish can be euthanized with a lethal dose of the agent applied to the gills [21]. In Brazil, MS-222 can be administered by various routes, water, gills, or lymphatic spaces and pleuroperitoneal cavities [28]. Further, when MS-222 is used in concentrations higher than 500 mg/L, the solution should be buffered. In Europe, anesthetic overdose can be used in fish where appropriate with prior sedation [29].

Euthanasia may occur by immersion in liquid nitrogen [30]; this technique is needed, for example, for the isolation of RNA and other preparations that preserve biological material from rapid degradation. In Brazil, this technique is accepted, with restrictions, on small fish (below 200 mg) [31].

Another method is immersion in water saturated with carbon dioxide, which causes narcosis and loss of consciousness, but acts slowly and is stressful for the fish. In Canada, the method with carbon dioxide in euthanasia is not accepted [27]. The American Veterinary Medical Association only accepts it if the source of CO_2 is precisely regulated, since the operator must take precautions on the contact with this gas [21]. In Brazil, this method should not be used in fish due to the acidity of the substance and prolonged maintenance of brain activity [31]. To European Union this method is not accepted also [29].

In general, physical methods should be complementary to other methods and can be used only if animals are unconscious. According to the Canadian Council on Animal Care, many fish still have brain activity even with hypoxia, then, only decapitation should be avoided, the fish brain should be frozen, pierced, or physically destroyed [27]. On the other hand, the American Veterinary Medical Association suggests decapitation followed by pithing and proceeded by anesthesia [21], as well as in Brazil, since the anesthesia is confirmed, it is accepted with restrictions [28]. As the central nervous system of fish is tolerant to hypoxia, pithing should be performed after the decapitation [31]. In Europe, use of concussion/percussive blow to the head is an accepted method and electrical stunning is acceptable if used specialized equipment [29].

For North-Americans, rapid chilling or hypothermic shock (2° C to 4° C) is accepted for the zebrafish until the loss of both orientation and opercular movement [21]. Unlike in Brazil, rapid chilling is not an accepted method, although it slows metabolism and eases handling, there is not any evidence that this process minimizes pain [31]. Rapid chilling may be applied to adults, larvae, and embryos, unlike maceration, which is only possible in adults because of their size. However, in hypothermic shock, one must take into consideration that when the temperature changes, *Danio rerio* cannot maintain its body temperature, creating a lethal heat stress [31].

Rapid freezing in non-anesthetized animals is an unacceptable method [21]. In Brazil, rapid freezing is accepted only when the animals are already anesthetized and therefore unconscious due to the formation of ice

crystals in their skin and tissues, which causes pain [31]. In contrast, some researchers demonstrated that, in adults, the rapid freezing is less stressful and more effective than MS-222 overdose; however, tissues contain ice crystals forming [32].

Practices that use animals to check benefits on human health should be better evaluated [33]. Therefore, it is necessary not only to consider the ethical principles of animal experimentation, but also consider the animals as a whole, taking into account their welfare, their physical and mental health, and that their death is less painful and distressing possible. Thus, methods of anesthesia and euthanasia in procedures and protocols of experiments with the zebrafish should be considered fundamental.

V. Conclusion

The purpose of using the animal model is to understand a particular disease without causing risk to a human being. Although in vitro cell culture assays are widely used, the results may not generate actual forecasts compared with in vivo results. Using animal testing is still necessary.

The search for new experimental models to reduce, refine, and replace the use of animal models has paved the way for new models, such as the *Danio rerio*, which has great similarity to mammals, therefore, it emerges as an important model for biomedical research.

Many attributes make the zebrafish an animal model of great importance, however, there is still much to discover about this species, and improve in welfare and methods of anesthesia and euthanasia. To further advance research with *Danio rerio*, more efforts are required, so that new information can flow to the understanding of biomedical research combined with the use of zebrafish.

We conclude that the concept of welfare is part of ethics in biomedical research. The possibility of using animals in research requires mental and physical health of the animals concerned. Therefore, more efforts for the advancement of biomedical research with zebrafish are fundamental.

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