ORIGINAL ARTICLE

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Use of ozone in the culture medium of zebrafish (*Danio rerio*) embryos

*Uso do ozônio no meio de cultura para embriões de zebrafish (*Danio rerio*)*

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ABSTRACT: In this study, we sought to demonstrate the toxicity of different concentrations of ozonated water in zebrafish embryos up to 120 hours postfertilization (hpf) and their antimicrobial activity advantages. For the test, we placed 40 embryos per treatment in Petri dishes containing 30 ml of solution to be tested. The ozone concentrations used were calculated from an initial concentration of 72 µg/ml using the O&L 1.5 RM ozone generator (Ozone & Life, Brazil). Five serial dilutions 1:1 was performed in egg water to produce the ozone treatments. We also analyzed a treatment with 30 ml of egg water without ozone, with only the addition of methylene blue, and a treatment with 30 ml of egg water without the addition of any antifungal agent. The plates were incubated at 28 ± 1°C, and the embryos were analyzed daily until 120 hpf. The survival rate, incubation period, and possible deformities were analyzed. In addition, egg water microbiological analyses were performed to detect total coliforms and fungi and yeasts. The data were tested for normality, and analysis of variance was performed within Minitab® version 1.8 software. The results showed that there was no significant difference in embryo survival or hatching rate between treatments with different ozone concentrations ($p > 0.05$), and no embryonic deformities were found under any of the ozone concentrations evaluated. It can be concluded that ozone within the concentrations analyzed is not toxic to zebrafish embryos and has antifungal and antimicrobial action.

KEYWORDS: Toxicity; ozone therapy; antimicrobial activity; antifungal activity.

RESUMO: Neste estudo, procuramos demonstrar a toxicidade de diferentes concentrações de água ozonizada em embriões de zebrafish até 120 horas pós-fertilização (hpf) e suas vantagens na atividade antimicrobiana. Para o teste, colocamos 40 embriões por tratamento em placas de Petri contendo 30 ml da solução a ser testada. As concentrações de ozônio utilizadas foram calculadas a partir de uma concentração inicial de 72 µg/ml usando o gerador de ozônio O&L 1,5 RM (Ozone & Life, Brasil). Foram realizadas cinco diluições seriadas 1:1 em Egg Water. Também analisamos um tratamento com 30 ml de Egg Water sem ozônio, apenas com adição de azul de metileno, e um tratamento com 30 ml de Egg Water sem adição de nenhum agente antifúngico. As placas foram incubadas a 28 ± 1°C e os embriões foram analisados diariamente até 120 hpf. A taxa de sobrevivência, período de incubação e possíveis deformidades foram analisados. Além disso, foram realizadas análises microbiológicas da Egg Water para detectar coliformes totais, fungos e leveduras. Os dados foram testados quanto à normalidade e a análise de variância foi realizada no software Minitab® versão 1.8. Os resultados mostraram que não houve diferença significativa na sobrevivência de embriões ou taxa de eclosão entre os tratamentos com diferentes concentrações de ozônio ($p > 0,05$), e não foram encontradas deformidades embrionárias sob nenhuma das concentrações de ozônio avaliadas. Pode-se concluir que o ozônio dentro das concentrações analisadas não é tóxico para embriões de zebrafish e possui ação antifúngica e antimicrobiana.

PALAVRAS-CHAVE: Toxicidade; ozonioterapia; atividade antimicrobiana; atividade antifúngica.

INTRODUCTION

The medical use of ozone, known as ozone therapy, is among the most promising oxidation therapies. It is an efficient technique that uses O_3 gas, which has great oxidative power and high potential for inactivation of microorganisms, including pathogenic bacteria present in the water, and it does not generate byproducts or any type of secondary pollution or toxic waste (PANDISELVAM et al., 2017). O_3 was discovered in 1840 by the German scientist Christian Friedrich Schoenbein. It is a molecule composed of three oxygen atoms

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in a dynamically unstable structure due to the presence of mesomeric states. Ozone therapy has been used and widely studied for more than a century, starting during the First World War. Its effects are proven, consistent, and safe, and it has minimal and avoidable side effects. Therefore, ozone therapy has been used to prevent and treat diseases. The mechanism of action is through the inactivation of bacteria, viruses, fungi, yeasts, and protozoa; the stimulation of oxygen metabolism; and the activation of the immune system (ELVIS; EKYA, 2011).

Due to these germicidal properties, ozone has been used in different fields, such as the food industry, wastewater treatment, and adjuvant treatment in veterinary therapy (PANDISELVAM et al., 2019; CHYS et al., 2018; CONSTANTIN; BIRTOIU, 2016). Rowen; Robins (2020) considered ozone therapy to be a very inexpensive and safe treatment modality. These authors emphasize that the technique can be implemented worldwide, even in very poor countries, to promote the prevention and treatment of acute and chronic diseases, such as COVID-19.

In aquaculture, its therapeutic use has not been widely explored. Ozonation is mostly used to disinfect water in recirculation systems. Ozone can play an important role in regulating water quality by decreasing the concentration of organic matter while not causing fish mortality (DE PAULA NASCENTE et al., 2019; SPILIOTOPOULOU et al., 2018). A growing number of studies demonstrate that the direct application of ozone can improve the productivity and welfare of fish species. Many of these observations seem to be correlated with pathogen reduction and/or water quality improvement. However, deleterious effects of direct ozonation have been observed, including behavioral abnormalities, changes in physiology, and tissue damage, when ozone has been used in fish farming systems (POWELL et al., 2018). Although the direct exposure of aquatic organisms to ozone and the oxidants produced may be lethal, different fish species can tolerate various levels of dissolved ozone. Thus, specific exposure levels need to be determined for each species, and reliable methods for measuring ozone in water are necessary to ensure that the lethal limits are not exceeded (GONÇALVES, 2011).

Among the many fish species currently known, *Danio rerio* (zebrafish) stands out for being a popular animal model in the fields of toxicology and biomedical research, making it a suitable animal for studying the possible effects of ozone on fish (CHAKRABORTY et al., 2016). Hao et al. (2015) established zebrafish as a new animal model for studies with ozone. However, to effectively discover the effects of ozonation in zebrafish, it would be necessary to ozonate egg water medium, a saline solution widely used to promote full early development of the species, both for maintenance in the laboratory and for control in embryotoxicity assays (ALI et al., 2011; ASAD et al., 2020). Therefore, studies involving the effect of ozone during the embryonic development phase in zebrafish have not been done.

In addition to the possible therapeutic advantages of ozone that can be investigated in zebrafish, its germicidal action can facilitate embryo maintenance and can be an alternative to methylene blue, a biocide currently included in egg water solution (DUMITRESCU et al., 2019). As reported, in recirculation systems, ozone is effective in controlling infectious diseases in marine and freshwater aquaculture, and proves to be safe for fish without the risk of waste contamination, unlike methylene blue that can remain in the environment. for a long period of time if not treated properly (JHUNKEAW et al., 2021; NASHMI et al., 2020). Therefore, the objective of this study was to evaluate the embryotoxicity to zebrafish of direct ozonation of egg water medium and the possible antimicrobial activity.

MATERIALS AND METHODS

Managing broodstock and facilities

All procedures followed the conventional guidelines for the care and use of laboratory animals of the *Conselho Nacional Brasileiro de controle de Experimentação Animal* (Brazilian National Council for Animal Experimentation control). All experimental procedures were registered by the animal ethics committee of the Federal University of Lavras (Brazil) (CEUA Protocol/045/2019). The experiment was conducted in the Fish Unit of the Central Animal Facility of the Department of Veterinary Medicine of the Federal University of Lavras, Minas Gerais, Brazil. The zebrafish (*Danio rerio*) broodstock, aged approximately 5 months, were kept in a rack (Alesco Hydrus Rack for Zebrafish) specifically designed for the species with a water recirculation system. The rack had 60 3-L tanks, and 10 animals were placed in each tank. Five male and five female fish were kept in each aquarium, separated by perforated acrylic partitions. The fish were kept under a 14:10 (light:dark) photoperiod (DAMMSKI et al., 2011) and at a temperature of 28°C and were fed (Nutrifish-Floculada fish food) three times a day.

Sample preparation

The ozone was dissolved in a specific medium for zebrafish embryo and larval development called egg water, which contains distilled water with salts that promote proper development. The egg water medium was composed of 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$ (ASAD et al., 2020). The ozone concentrations used were calculated from an initial concentration of 0.47 µg/ml, maximum concentration provided by the equipment used, the O&L 1.5 RM ozone generator (Ozone & Life, Brazil) and also because it is the recommended concentration for the practice of ozonation

of Water (PEÑA et al., 2017). The methodology applied to measure the ozone quantity was based on Gottschalk et al. (2009). Five serial dilutions were performed in egg water to obtain the ozone treatments:

- Treatment 1: 30 ml of ozonated egg water at the initial concentration (0.47 µg/ml of ozone);
- Treatment 2: 25 ml of ozonated egg water at the initial concentration was added to 5 ml of egg water without ozonation (0.39 µg/ml of ozone);
- Treatment 3: 20 ml of ozonated egg water at the initial concentration was added to 10 ml of egg water without ozonation (0.31 µg/ml of ozone);
- Treatment 4: 15 ml of ozonated egg water at the initial concentration was added to 15 ml of egg water without ozonation (0.23 µg/ml of ozone);
- Treatment 5: 10 ml of ozonated egg water at the initial concentration was added with 20 ml of egg water without ozonation (0.15 µg/ml of ozone);
- Treatment 6: 5 ml of ozonated egg water at the initial concentration was added to 25 ml of egg water without ozonation (0.07 µg/ml of ozone);
- Treatment 7: 30 ml of egg water without ozonation with the addition of methylene blue antifungal, constituting the positive control group;
- Treatment 8: 30 ml of egg water without the addition of any antifungal agent, constituting the negative control group.

Method of obtaining embryos

Five males and five females of zebrafish adults were separated according and kept in three-liter tanks with acrylic dividers (Alesco Hydrus Rack). Three breeding aquariums were placed, with the same number of animals, to obtain the embryos necessary for the analysis. They were kept at a temperature of 28°C under a 14:10 photoperiod. Breeding occurred in the morning, in the first hours of light, when the males and females of each tank were placed together in a zebrafish-specific breeding tank. The animals remained together for 2 hours and then were returned to the rack, with males and females being separated. The embryos were collected through a siphon at the bottom of the breeding tanks and kept in egg water medium in Petri dishes in an incubator at 28°C.

Embryotoxicity test

To assess the embryotoxicity of ozonated water, the embryos at 5 hours postfertilization (hpf) were placed in Petri dishes containing 30 ml of solution. A total of 40 embryos were used per treatment, and the assay was performed in triplicate. During exposure, the Petri dishes were incubated at 28°C in a Thermo Scientific Forma 311 incubator. To observe survival rate, hatching rate, and possible morphological abnormalities, the embryos were evaluated daily up to 120 hpf according to the embryotoxicity protocol recommended by the OECD (2013). The following embryonic characteristics were considered morphological abnormalities: absence of somite formation or eye development, degree of pigmentation, presence of edema during heart formation, no detachment of the tail, lack of heartbeat, growth retardation, lordosis, and tail deformities (HALLARE et al. 2006). Based on the normal parameters for the species (KIMMEL et al. 1995), any other characteristics that did not correspond to normal zebrafish development were considered morphological abnormalities. The animals were evaluated using a microscope (Olympus, model CX31) with a 4x magnification. All experimental procedures, both for breeding and for toxicological tests, followed the specifications of the Organization for Economic Cooperation and Development (OECD, 2013).

Microbiological analysis

After the embryotoxicity test, which lasted 120 hours, the water samples from each treatment were kept for the following analyses:

Bacteria quantification analysis: To determine the presence of coliform bacteria, the most-likely-number (MLN) technique was performed following the method of Hunt; Rice (2005). For the coliform test, lauryl sulfate tryptose broth was used as the medium in test tubes containing Durham tubes. For each of the eight treatments, three series with three tubes each were used.

For the first series, 1 ml of the crude sample was transferred to each of three tubes containing 10 ml lauryl sulfate tryptose broth. For the second series, 1 ml of the 10^{-1} dilution was transferred to each tube. For the third series, 1 mL of the 10^{-2} dilution was transferred to each tube. The dilutions were made in saline solution.

The tubes were incubated at 34°C for 48 hours. The tubes that showed turbidity and gas inside the Durham tubes were considered positive for coliforms. The tubes that did not show turbidity were considered negative. The results are expressed in MLN/100 mL, based on the number of positive tubes.

Determination of the presence of fungi and yeasts: Petri dishes containing approximately 20 mL of Sabouraud agar were used. For each of the eight treatments, 10^{-1} and 10^{-2} dilutions in saline were performed. The analysis was performed in triplicate for each dilution, totaling 72 samples for the entire assay. The samples were spread on Sabouraud agar plates using Drigalski spatulas in a completely sterile environment. The plates were kept in the Microbiology Laboratory of the Department of Veterinary Medicine of UFLA and incubated at room temperature for 15 days. On that occasion, the colony forming units of fungi and yeasts were counted, and the values were statistically compared.

Statistical analysis

The data were tested for normality using the Shapiro-Wilk test. Since all data were normally distributed, analysis of variance (ANOVA) was performed in Minitab® software version 1.8.

RESULTS

Embryotoxicity

Initially, to evaluate the potential toxicity of ozone in the embryonic development of zebrafish, some common developmental phenotypes were analyzed, including the survival and hatching rates. The different ozone concentrations tested did not induce lethality or teratogenicity in zebrafish, as no deformities were found during the analyses (Figure 1). There was no significant difference in survival or hatching rate between groups ($p > 0.05$) (Table 1).

Microbiological analysis

Coliform bacteria were detected in 90% of the samples, with populations ranging from a MLN of 2.5×10^4 to more than 140×10^4 total coliforms/ml (Table 2). The concentration of 0.07 µg/ml of ozone was the only concentration in which there was no presence of coliforms.

In the analysis performed to observe the presence of no statistical difference between treatments with the positive control (Table 3) .

DISCUSSION

In the present study, the effect of ozone on zebrafish embryos was evaluated by morphological and microbiological analyses. The results showed that ozone at the tested concentrations did not cause lethality or abnormalities during the embryonic development of this species. The survival data show that the ozone treatments and the positive and negative control treatments all let zebrafish early development proceed as usual, with a spontaneous mortality rate between 5 and 25% (ALI et al., 2011). These results show that zebrafish embryos exhibit considerably high tolerance to ozonation. Hao et al. (2015) determined that the safe concentration of ozonated water for zebrafish embryos was 0.1 mg/L and reported that this concentration allowed survival and did not harm the animals. Resistance to ozonation was also found by other authors in embryos of marine fish such as *Gadus morhua* and *Hippoglossus hippoglossus*, being 3 mg/L and 2 mg/L respectively (FRY et al., 2015; GROTMOL et al., 2003). The embryos of the marine fish species *Hippoglossus hippoglossus* showed greater sensitivity to ozonation (GROTMOL et al., 2003). These data indicate

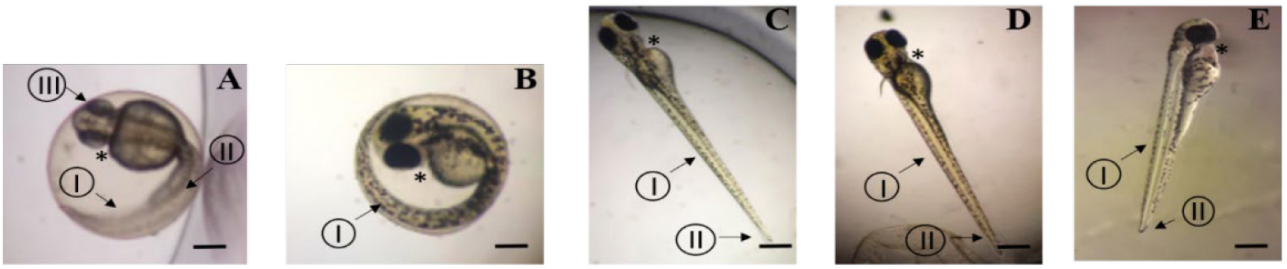

Figure 1. Morphological characteristics found in all treatments during the embryotoxicity and teratogenicity assay at different ozone concentrations. *Absence of edema in the pericardium and/or yolk sac. 1A: I - detachment of the tail; II - formation of somites; III - eye formation; 1B: I - presence of pigmentation; 1C, 1D and 1E: I - absence of lordosis; II - absence of tail deformity. Bar = 100 µm.

Table 1. Survival and hatching rates of zebrafish embryos. Values are mean ± standard error of the mean

Treatment	Survival (%)	Hatching (%)
0.47 µg/ml of ozone	87.50 ±2.50	87.50±2.50
0.39 µg/ml of ozone	93.75 ± 6.25	81.25±6.25
0.31 µg/ml of ozone	87.50±2.50	90.00±0.1
0.23 µg/ml of ozone	96.25±1.25	79.3±13.20
0.15 µg/ml of ozone	87.50±2.50	85.00±5.0
0.07 µg/ml of ozone	87.50±2.50	82.50 ±7.50
Positive control	90.25±0.2	85.00 ±5.0
Negative control	95.00±0.1	85.00 ±5.0

Table 2. Most likely number (MLN) of total coliforms per milliliter of water.

Treatment	Sample (MLN/ml)
0.47 µg/ml of ozone	> 140 x 10^4
0.39 µg/ml of ozone	110 x 10^4
0.31 µg/ml of ozone	9.5 x 10^4
0.23 µg/ml of ozone	9.5 x 10^4
0.15 µg/ml of ozone	7.5 x 10^4
0.07 µg/ml of ozone	Absent
Positive control	2.5 x 10^4
Negative control	9.5 x 10^4

Table 3. Absolute number of fungi and yeast colony-forming units.

Treatments \ Dilutions	Fungi			Yeast		
	10^{0}	10^{-1}	10^{-2}	10^{0}	10^{-1}	10^{-2}
0.47 µg/ml of ozone	6±6A	0±0A	0±0A	1±1 A	0±0 A	0±0 A
0.39 µg/ml of ozone	7±6,4 A	0±0 A	5±1 A	0±0 A	0±0 A	0±0 A
0.31 µg/ml of ozone	2±6,3 A	2±1 A	0±0 A	1±0,57 A	0±0 A	0±0 A
0.23 µg/ml of ozone	1±0 A	0±0 A	0±0 A	0±0 A	0±0 A	0±0 A
0.15 µg/ml of ozone	1±0,5 A	2±2 A	0±0 A	0±0 A	0±0 A	0±0 A
0.07 µg/ml of ozone	1±2,3 A	0±0 A	0±0 A	0±0,5 A	0±0 A	0±0 A
Positive control	0±0 A	1±0,5 A	1±0,5 A	0±0 A	0±0 A	0±0 A
Negative control	34±15 B	15±7,6 B	0 ±0 B	0±0	1±1	0±0

Means followed by equal capital letters do not differ statistically between treatments for each group ($P<0.05$). Analyzes performed by the Tukey test.

that embryos of different fish species have different tolerances to ozone.

The different concentrations of ozonated egg water also did not cause delays or decreases in hatching rate compared to the positive control egg water. Such delays or decreases have been reported as side effects of ozonation in some marine fish species, such as *Latris lineata* (BATTAGLENE; MOREHEAD 2006). On the other hand, as demonstrated in zebrafish here, *Oncorhynchus mykiss* and *Hippoglossus hippoglossus* embryos have shown satisfactory hatching and growth results in ozonated water (FRY et al., 2015).

Our results also demonstrated that 0.07 µg/ml of ozone fully sterilized the environment against coliform bacteria, fungi, and yeasts. This concentration 0.07 µg/ml of ozone had better action than the positive control group, demonstrating that the use of methylene blue cannot fully sterilize the solution against fungi and yeasts. Our data also demonstrate the importance of using an antifungal agent in zebrafish embryo water media because the negative control group had the most fungal and yeast colonies. These results demonstrate the great antimicrobial and antifungal potential of ozone. Ozone may therefore help reduce the propagation and transfer of pathogens between the offspring during the incubation period. This is because ozonation has good sterilization properties, is safer and more potent than conventional disinfectants, and acts on a wide variety of microorganisms, including resistant pathogens, without producing toxic byproducts or waste (CARDOSO et al., 2003).

The factor that makes ozone an excellent antifungal agent is its strong oxidizing property (MOHAMMAD et al., 2010). Because ozone can produce oxidative stress, its action is related to the potential to form reactive oxygen species (ROS) inside fungal cells. ROS can cause damage to their cellular compounds and lead to cell dysfunction or cell death (ONG; ALI 2015).

The antibacterial action of ozone results from its high reactivity and great oxidizing power. Therefore, its bacterial inactivation may be due to cell envelope damage or breakdown, which leads to leakage of cell contents and cell lysis (GREENE; GUZEL-SEYDIM; SEYDIM, 2012). The destructive action of ozone on bacteria involves successive damage to cell walls, the cytoplasmic membrane, and, finally, the DNA structure of the bacterial cell, resulting in the inability to resist an ozone attack (OIZUMI et al., 1998; BRODOWSKA; NOWAK; ŚMIGIELSKI, 2018).

Other studies have also shown that ozonation resulted in beneficial decreases in the bacterial and fungal loads in embryos and larvae of fish species such as *Dicentrarchus labrax*, *Oncorhynchus mykiss*, *Psetta maxima*, *Acanthopagrus schlegelii*, and *Melanogrammus aeglefinus* (CAN et al., 2012; DAVIDSON et al., 2011; POWEL et al., 2015; KEVIN et al., 2006). However, there are still few studies on the effects of ozonation on freshwater fish embryos, as mentioned in the study carried out by JHUNKEAW et al. (2021) in which they evaluated ozone toxicity in tilapia embryos.

The lowest concentration of ozone tested also had similar results to the highest concentrations in the study by Fagundes (2013), in which the ozone concentration of 0.5 ppm had statistically equal bactericidal and antifungal action than the highest concentrations of ozone tested (1, 0 and 1.5 ppm). Knowing the lowest effective concentration of ozone in egg water is important because egg water contains NaCl. According to Bocci (2005), ozonized NaCl leads to the formation of hypochlorous acid. Thus, a greater amount of ozone can form substances that prevent it from performing its function. These current and previous data are interesting because they demonstrate that there is no need for high concentrations of ozone to kill bacteria and fungi, so effective ozonation is unlikely to be toxic to animals.

CONCLUSION

The tested concentrations of ozone in Zebrafish egg water did not cause lethality or teratogenicity in zebrafish embryo, demonstrating the resistance of the species to ozonation. This is the first embryo egg water ozonation study in Zebrafish suggesting that ozone has great potential to solve problems in aquaculture. Although we have confirmed its biocidal potential,

additional studies will be needed to elucidate the therapeutic applications of ozonation, both in fish production and for fish health.

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