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ORIGINAL ARTICLE

Morphological characterization of Artemia salina in its different developmental cycles and its application in biocompatibility testing

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ABSTRACT

Background: Artemia Salina is a tiny crustacean that can tolerate high salt concentrations and survive in low-oxygen conditions. It is sexually dimorphic, meaning it has both sexual and asexual life cycles. In its adult stage, it measures between 17-18 mm in length and is used in aquaculture and toxicity testing.
Objective: To determine the most suitable stage of development of Artemia Salina for biocompatibility testing.
Materials and methods: An in vitro experimental study was conducted using Artemia salina as a biomodel. Detailed observations were made of the life cycle of Artemia salina, from its cyst stage to adulthood.
Results: A culture and feeding protocol was established for its maintenance in the laboratory. Common dental biomaterials were used to evaluate their cytotoxicity in Artemia larvae.
Conclusion: Toxicity tests with dental biomaterials showed that both calcium hydroxide and zinc oxide-eugenol were toxic to Artemia larvae, with a mortality rate of 100% within the first 24 hours.

CLINICAL RELEVANCE

The study provides detailed information on the life cycle of Artemia Salina and its maintenance in the laboratory to determine at which stage of development it is best to capture it in order to obtain valid and replicable results.

INTRODUCTION

Artemia salina is a primitive arthropod that was first reported in Lake Urmia in 982 BC. It has a slender, segmented body covered with chitin, which is brownish in color and transparent to light, and grows to an adult size of about 8-12 mm in length. Also known as the "sea monkey," it can tolerate large amounts of salt (up to 300 grams of salt per liter of water) and survive in waters with high oxygen deficiency. It exhibits sexual dimorphism and can be oviparous and/or ovoviviparous.

Used mainly as a food source in fish farming and aquaculture, this arthropod has proven to be a valuable biomodel for cytotoxicity testing, mainly in ethnomedicine and ecotoxicology, to determine the effects of medicinal plants on living beings. Since 1980, standardization for this type of test has been underway, and its applicability as a valid preliminary method for initial toxicity screening has been observed in the fields of nanotechnology and dentistry, demonstrating that its implementation can be carried out without special infrastructure or equipment requirements. has been observed in the field of nanotechnology and in the area of dentistry, demonstrating that its implementation can be carried out without special infrastructure or equipment requirements, is economical, easy to handle, and has a high capacity for adaptability.

MATERIALS AND METHODS

Type of research

In vitro experimental study using an invertebrate animal biomodel. To carry out this research, the life cycle of Artemia salina was evaluated and studied for more than 60 hours in media adapted for its development. In addition, a feeding protocol was implemented for its maintenance in the laboratory and to enable toxicity testing throughout its life.

Instruments and procedures

Based on the protocol "STANDARDIZATION OF A BIOCOMPATIBILITY PROTOCOL WITH ARTEMIA SALINA IN DENTAL BIOMATERIALS",[2] Artemia salina cysts were hydrated, taking into account that 1 gram of Artemia salina cysts is added for each liter of artificial seawater solution. and to prepare this solution, 35 grams of sea salt were mixed into 1 liter of distilled water using magnetic stirring.

To hatch the cysts, an artemizer manufactured by the authors of the project entitled "Standardization of a biocompatibility protocol with Artemia salina" was used, which was based on the equipment used by Rotini et al. [3] which consists of a series of concentric rings that act as barriers to ensure that only healthy nauplii reach the central ring where they will be captured for the experiment.[2]

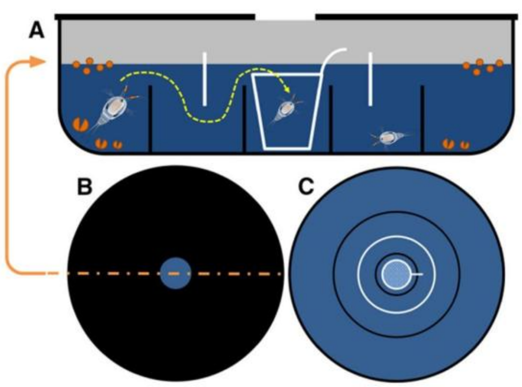

Figure 1. Barriers to ensure that only healthy nauplii reach the central ring

The foods selected for the Artemia salina maintenance studies were: Active Dry Yeast (Levapan, Bogotá, Colombia), Natural Wheat Bran (Toning, Yumbo, Colombia), and Spirulina (Yumbo, Colombia). The amount of food used was determined using a rule of three, which yielded 2 mL of artificial seawater/2 mg of yeast; 4 2 mL of artificial seawater/200 mg of wheat bran; 5 and 1 L of artificial seawater/400 mg of spirulina.

The foods were prepared at 25°C by grinding and diluting them in artificial seawater solution, and they were mixed by magnetic stirring. A volume based on the size of the wells in the microplates was used.

To carry out the experiments, 24-well Coster plates were used, of which only 15 wells were used to deposit 2 mL of solution of each food and negative and positive control groups. For each well, 5 nauplii of 48 hours were deposited; Artificial seawater solution was used for the negative control, and enzymatic soap (Bonzyme, Laboratorios Eufar, Bogotá, Colombia) was used for the positive control, in order to obtain mortality data and determine a protocol for the maintenance of Artemia salina. Additionally, 6-well Coster plates were used, in which 10 mL of each food was deposited in different concentrations and 10 nauplii aged 48 hours. The negative control group was only taken into account to evaluate the adaptability of Artemia in media with different food concentrations.

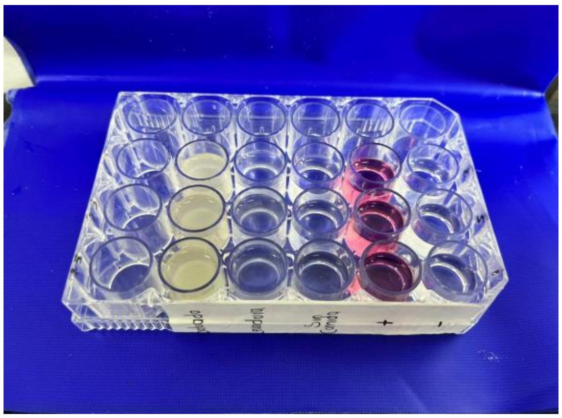

Figure 2. Food distribution and control groups in 24-well culture plates. Source: authors' own.

The dental biomaterials selected for the study were calcium hydroxide (CaOH) paste (Dycal®, Dentsply Sirona, Milford, DE, USA), which is used for indirect pulp capping in vital therapies; and zinc oxide-eugenol (Zinc Oxide® Prodont Scientific, Bogotá, Colombia), used as a sealing cement for temporary fillings.

The materials were prepared at 25°C according to the manufacturers' instructions. For the proper development of this research, four main phases were agreed upon in order to meet our objectives:

- Phase 1: Literature Review.
- Phase 2: Morphological Characterization of Artemia Salina.
- Phase 3: Cultivation, Maintenance, and Feeding of Artemia Salina.
- Phase 4: Cytotoxicity Assessment.

During phase 1, articles on platforms such as Scopus or Medline related to the life cycle of Artemia Salina and how it is used for toxicity studies in different areas of study will be reviewed.

Phase 2 consists of studying the developmental stages of Artemia Salina chronologically, where the hatching process will be carried out using equipment based on Rotini et al,[3] dividing the hatcher into zones Z1, Z2, and Z3 corresponding to the peripheral, middle, and center areas. Artemia Salina were then captured from each zone at 6, 12, 24, 36, 48, and 60 hours after cyst hydration, and photographs were taken during each capture to observe each stage of Artemia Salina development. The development of this phase was carried out with the aid of optical microscopy, histology techniques, and scanning electron microscopy.

For phase 3, the objective of this phase is to maintain a permanent culture of Artemia salina for at least three months in order to carry out cytotoxicity studies at different stages of A. salina development. Salina. In this phase, the hydration stage was carried out according to the protocol of "Standardization of a biocompatibility protocol with artemia salina in dental biomaterials".[2] Artemia Salina is captured at 6, 12, 24, 36, and 48 hours in 6x4 Coster plates and distributed into groups: positive, negative, no food, yeast food, wheat bran food, and microalgae food, in order to obtain a long-term artemia culture with a sustainable feeding protocol.

In phase 4, the cytotoxicity of dental biomaterials is evaluated. For this purpose, the two most toxic biomaterials (calcium hydroxide and zinc oxide) were selected from the research project "STANDARDIZATION OF A BIOCOMPATIBILITY PROTOCOL WITH ARTEMIA SALINA," in which A. Salina was captured at different stages of development and placed in 6-well plates where the groups in contact with the material to be evaluated, positive control, and negative control were located. Subsequently, the toxicity of the material was evaluated after 24 and 48 hours, determining the reliability of the toxicity tests based on a mortality formula.[6,7] In addition, a constant rate of at least 10% mortality or less will be maintained in the negative control, and a material will be classified as toxic when a mortality rate greater than or equal to 30% is obtained.[8]

Mortality % = (Number of dead AS / Initial number of AS) x 100

Figure 2. Mortality formula.

RESULTS

For phase 2 of the morphological characterization of Artemia salina, the hydration protocol [2] was performed, and a visual inspection was carried out after 6 hours under a stereomicroscope at different magnifications (1X, 2X, 3X, 4X), where A. Salina in the hydrated cyst phase, where the increase in cyst size could be visualized.

Figure 3. Hydrated cyst. Source: authors' own. Image taken with a stereomicroscope at 4X magnification.

Twenty-four hours after hydration, the E1 rupture phase was observed, evidenced by the presence of a crack around its circumference.

Figure 4. Cyst in rupture phase E1. Source: authors' own. Image taken with a 4X stereomicroscope.

At 36 hours, the cyst can already be seen in the rupture phase E2, in which part of the A. Salina can be seen hatching inside its yolk sac. A. Salina already in the complete hatching phase were also observed, however, they are still enclosed in their yolk sac.

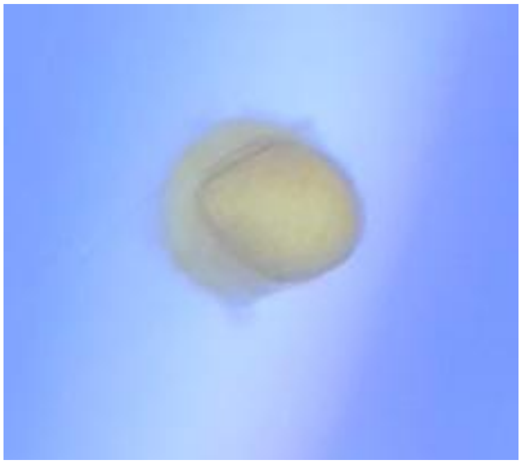

Figure 5. Cyst in the rupture phase E2. Source: authors' own. Image taken with a stereomicroscope at 4X magnification.

Figure 6. Cyst in the hatching phase. Source: authors' own. Image taken with a 4X stereomicroscope.

Subsequently, at 48 hours, A. Salina in the nauplius I stage, which, despite having been completely released from the cyst, is still enclosed in its yolk sac, making it impossible to differentiate its morphological parts. Similarly, A. Salina in the nauplius II stage, already in the phase of complete release from the yolk sac, can be observed, displaying the characteristic carmelite color of this biomodel test.

Figura 7. A. Salina en fase de Nauplio I. Fuente propia de los autores. Imagen tomada en estereomicroscopio a 4X.

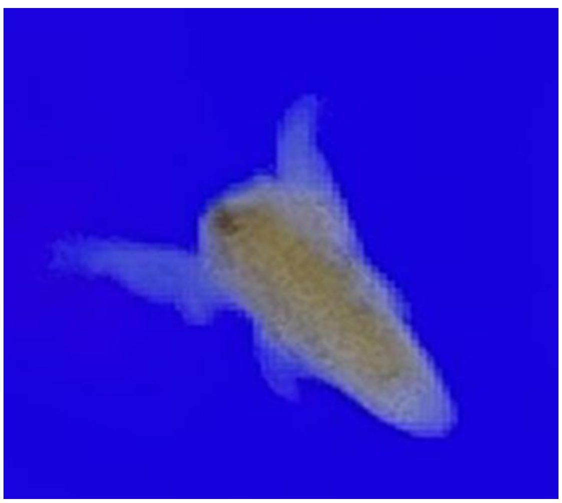

Figure 8. A. salina in the Nauplius II stage. Source: authors' own. Image taken with a 4X stereomicroscope.

Approximately 60 hours after hydration, it is possible to find Artemia in the metanauplius stage, which can be distinguished by the fact that most of their bodies are transparent but without completely losing their initial color. In this same visualization, Artemia in the juvenile and adult stages were found, which can be distinguished by being completely transparent. However, it can be observed that young artemia have only one nauplius eye, while adults have three nauplius eyes and a more complex structure. In the adult stage, it is possible to differentiate them by sex, whereby we observe greater complexity in the structure of the female artemia than in the anatomy of the male artemia.

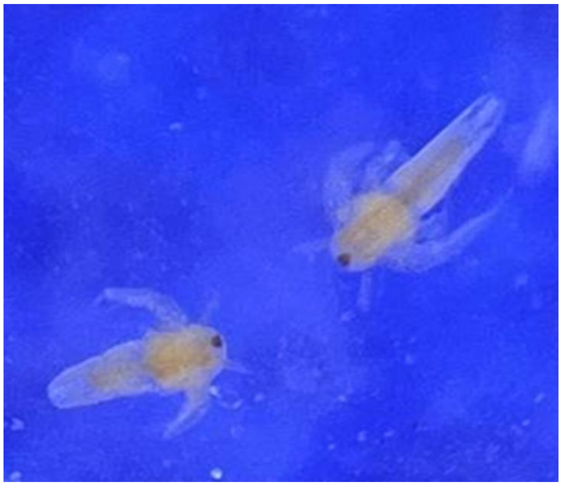

Figure 9. A. Salina in the metanauplius stage. Source: authors' own. Image taken with a stereomicroscope at 4X magnification.

Figure 10. A. Salina in juvenile stage. Source: authors' own. Image taken with a stereomicroscope at 4X magnification.

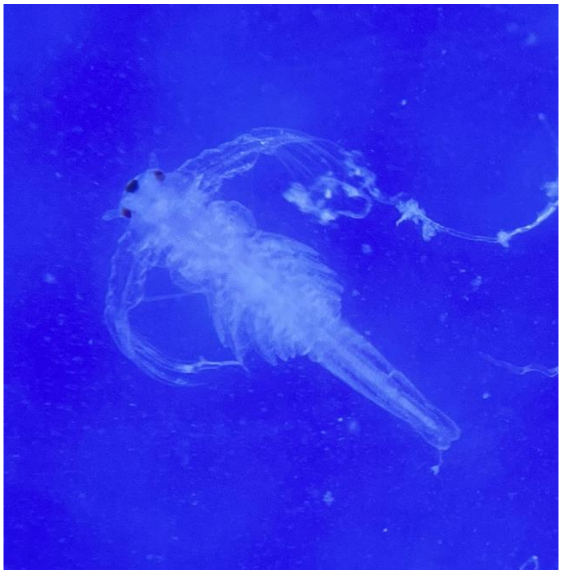

Figura 11. A. Salina en fase Adulta. Fuente propia de los autores. Imagen tomada en estereomicroscopio a 4X.

Figure 12. Female Artemia Salina. Source: authors' own. Image taken with a stereomicroscope at 4X magnification.

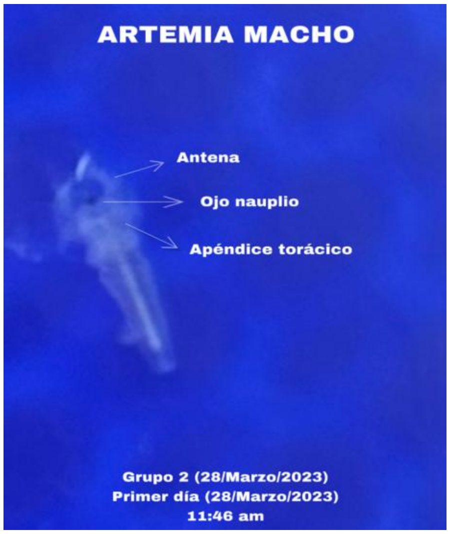

Figure 13. Male Artemia Salina. Source: authors' own. Image taken with a stereomicroscope at 4X magnification.

For phase 3 of Cultivation, Feeding, and Maintenance of A. Salina, A. Salina was cultivated, three foods were selected based on the literature and prepared separately as follows: 200 mg of wheat bran/2 ml of artificial seawater; 2 mg of yeast/2 ml of artificial seawater; 0.7 g of spirulina/500 ml of artificial seawater.

Subsequently, observations were made under a 4X stereoscopic microscope to evaluate the mortality rate with each feed, for which no favorable results were obtained due to environmental conditions and probably the amount of feed provided, so it was decided to prepare the feeds in different concentrations, using the initial concentrations and new ones at lower concentrations. Observations were made and it was found that wheat bran generated a high mortality rate, so it was decided to exclude it from the experiment and continue testing with more variations in yeast and spirulina concentration: high yeast concentration (40 ml/42 mg), medium yeast concentration (40ml/14mg), low yeast concentration (40ml/6.6mg), high spirulina concentration (500ml/0.7g), low spirulina concentration (500ml/0.35g). Observations and mortality rates show that A. Salina has a lower mortality rate when fed yeast.

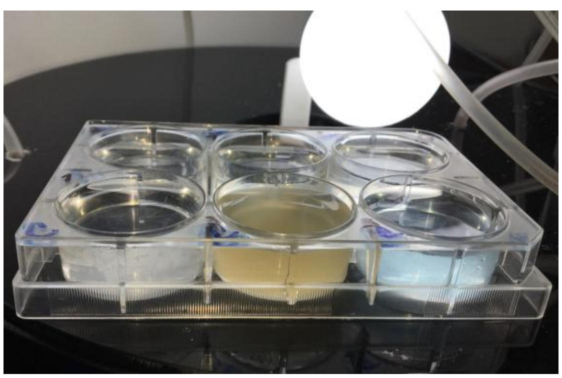

Figure 14. Group with foods at different concentrations and negative control group. Source: authors' own.

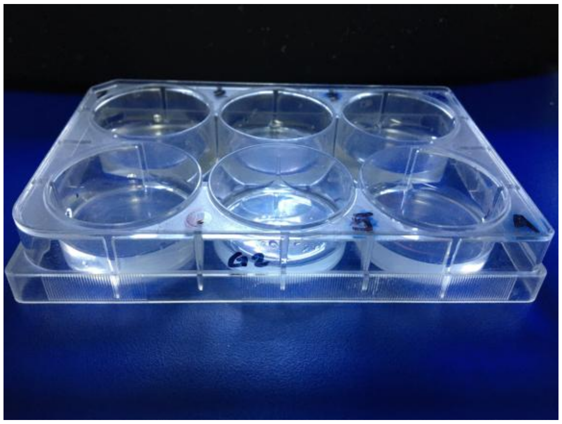

Figure 15. Group with yeast and spirulina in different concentrations and negative control group. Source: authors' own.

Observations were also made of Artemia Salina without any food, which survived for 20 days, showing that they feed on the yolk sac of dead artemia.

Figure 16. Adult female Artemia salina 20 days after hatching. Source: authors' own. Image taken with an optical microscope at 10x magnification.

For phase 4 of the toxicity assessment, cytotoxicity was evaluated with two dental biomaterials, calcium hydroxide and zinc oxide-eugenol, materials widely used in dentistry for various purposes and patient management. Four groups of 10 Artemia larvae were captured 36 hours after the cysts were hydrated. Each group was classified as group 04 (positive control being enzymatic soap), group 03 (negative control being artificial seawater), group 2 (artemia that had contact with the material after it had set), and group 1 (artemia that had immediate contact with the material after it had been spatulated). It should be noted that the two materials used in this test were prepared and spread according to the manufacturer's instructions. Each group of 10 brine shrimp was placed in a well of coast plates with 2 ml of artificial seawater, the cytotoxicity test was performed, and the following results were obtained:

Group 1	Group 2	Group 3	Group 4
Immediate contact after spatulation of CaOH	Artemias had contact 1 hour after spatulation	Control with artificial seawater	Control with enzymatic soap
24 hours: 100% mortality	24 hours: 100% mortality	24 hours: 40% mortality (6 live with good motility)	24 hours: 100% mortality
48 hours: 100% mortality	48 hours: 100% mortality	48 hours: 40% mortality (4 live with good motility, 2 live with reduced motility)	48 hours: 100% mortality

Figure 17. Cytotoxicity results with calcium hydroxide.

Figure 18. Live Artemia Salina in a well with calcium hydroxide. Images taken with a stereomicroscope at 4x magnification. Source: authors' own.

During this cytotoxicity test with CaOH, it was found that both groups 1 and 2 had a 100% mortality rate at 24 and 48 hours, resulting in a toxic material.

Group 1	Group 2	Group 3	Group 4
Immediate contact after spatulation of zinc oxide - eugenol	Artemias had contact 1 hour after spatulation of zinc oxide - eugenol	Control with artificial seawater	Control with enzymatic soap
24 hours: 100% mortality	24 hours: 100% mortality	24 hours: 30% mortality (7 live with good motility)	24 hours: 100% mortality
48 hours: 100% mortality	48 hours: 100% mortality	48 hours: 60% mortality (4 live with good motility, 2 live with reduced motility)	48 hours: 100% mortality

Figura 19. Resultados de citotoxicidad con Óxido de zinc-eugenol.

Figure 20. Live Artemia Salina in a well with zinc oxide-eugenol. Images taken with a 4x stereomicroscope. Source: authors' own.

In the cytotoxicity test with zinc oxide-eugenol, groups 1 and 2 had 100% mortality in the first 24 hours, concluding that it is a toxic material for testing with Artemia salina.

DISCUSSION

Research on Artemia Salina provides a structured development cycle of this invertebrate biomodel, specific morphological characteristics of each stage, and its sexual differentiation. The in vitro experimental study details the culture and feeding protocol necessary for the maintenance of Artemia in the laboratory, as well as the use of common dental materials to evaluate their cytotoxicity. The results reveal that both calcium hydroxide and zinc oxide-eugenol were toxic to Artemia larvae, with a mortality rate of 100% within the first 24 hours.

In summary, this study highlights the importance of Artemia Salina as a biomodel for evaluating the toxicity of materials, especially in the field of dentistry and scientific research. It provides specific information on its life cycle, feeding, and maintenance in the laboratory and establishes a protocol for future research in this field. The findings highlight the need to consider the specific stage of Artemia Salina capture in order to obtain suitable and accurate results.

CONFLICT OF INTEREST AND SOURCE OF FUNDING

This research was financially supported by the Dean of Research of the Faculty of Health at the Universidad del Valle, the School of Dentistry at the Universidad del Valle, and the authors' and tutor's own sources.

STATEMENT ON ETHICAL ASPECTS

Artemia salina larvae, an invertebrate organism, were used in this research. The procedures were carried out in accordance with those recommended in the scientific literature [3,7] and the protocols established by the Dental Biomaterials Research Group. The study was reviewed and authorized by the Animal Research Ethics Committee of the Universidad del Valle under code CEAS 002-021.

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