ORIGINAL ARTICLE

Exploring the Impact of Triple Antibiotic Paste and Nanosilica- Triple Antibiotic Paste on the Embryonic Development Stages of Zebrafish

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Abstract

Background: Triple Antibiotic Paste (TAP) is widely used as an intracanal medicament in endodontics. With the introduction of nanosilica-based formulations (Np-TAP) to enhance delivery and efficacy, concerns regarding their potential environmental and developmental toxicity have emerged. **Aim:** To evaluate the developmental toxicity of TAP and nanosilica-based TAP (Np-TAP) on zebrafish (Danio rerio) embryos by assessing hatching rates and viability. **Materials and Methods:** Zebrafish embryos were exposed to TAP and Np-TAP at concentrations ranging from 10 to 160 µL from 24 to 96 hours post-fertilization. Untreated embryos served as controls. Embryonic development, hatching rates, viability, and morphological changes were assessed at 24-hour intervals. **Results:** Both TAP and Np-TAP induced dose-dependent delays in hatching. Lower concentrations demonstrated complete recovery of hatching by 48 hours, whereas higher concentrations significantly inhibited hatching, with complete absence observed at the highest doses by 96 hours. Embryo viability decreased progressively with increasing concentration and exposure duration, despite initially being 100% across all groups. Incorporation of nanosilica did not reduce the developmental toxicity associated with TAP. **Conclusion:** High concentrations of TAP and Np-TAP adversely affect zebrafish embryonic development, indicating potential ecological risks. The addition of nanosilica did not mitigate TAP-induced toxicity. These findings emphasize the need for precise dosing in endodontic applications and warrant further environmental risk assessments of antibiotic-containing nanomaterials.

Key words: Hatching rate, public health, silica nanoparticle, triple antibiotic paste, zebrafish embryos

INTRODUCTION

The zebrafish (*Danio rerio*) has been identified as a phenomenal organism close to the human's ecological system, being used as a laboratory model in various research studies. Its excellence falls in genetic malleability, ease of reproduction, and the remarkable transparency of its embryonic stages.[1] Zebrafish larvae hold a unique specification in high-throughput drug testing, which helps the researchers to test combinations, such as small size, optical translucence, and prolific embryo production.[2] The applicability of zebrafish is very extensive, ranging from scientific domains, encompassing toxicology, early-life exposure to xenobiotics, disease modeling, and screening for bioactive compounds.[3] Considering cancer research, zebrafish plays a pivotal role by being an invaluable model as it is ideal for analyzing the process of carcinogenesis, tumor progression, metastasis, and drug resistance.[4] In addition to cancer research, they are utilized in the domain of aquatic ecotoxicology by analyzing the toxicity of environmental pollutants along with bioaccumulation which ideally helps researchers to monitor the consequences of pollution in aquatic ecosystems.[5]

Assessing the various embryonic stages of zebrafish throughout the study has been a challenge, but a breakthrough was made by the imaging techniques that were invented, such

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as optoacoustic mesoscopy and selective plane illumination microscopy. The fusion technology identifies through the developmental stages of zebrafish, starting from the embryonic phase to adulthood. The combination of these two techniques is a novel method that enhances precision and paves the way for more incorporation of technological advancements in the field of research.[6]

There are numerous antimicrobial medicaments, but certain medicaments are being consistently studied in the past decade by various endodontists as they both possess tremendous properties. Those include triple antibiotic paste (TAP), which is a combination of three antibiotics, including metronidazole, ciprofloxacin, and minocycline proven to be an exceptional therapeutic agent in eliminating pathogens.[7] Nanosilica-based TAP (Np-TAP), a nanosilica-based counterpart of TAP, is a new innovation to the conventional therapies where nanosilica, such as doxycycline, flagyl, and ciprofloxacin, are incorporated into the drug delivery system. These medicaments are being consistently studied in the past decade by various endodontists as they both possess tremendous properties.[8,9] This invention brought the mechanism of having controlled and sustained antimicrobial agent release, potentially amplifying its effectiveness.[10]

The antimicrobial efficacies of TAP and Np-TAP have been compared with other medicaments that are used for intracanal dressings have been studied by various endodontic researchers. Studies have concluded about the superiority of TAP in comparison to plain calcium hydroxide (CH) and *Ocimum sanctum*, highlighting its efficacy as an antimicrobial agent. Nevertheless, no significant divergence was noted between TAP and CH plus 2% chlorhexidine.[11] However, the efficacy through clinical applications of TAP need further clinical trials.

These trials are imperative to secure robust clinical evidence, ensuring the endorsement and widespread use of TAP in the realm of endodontic practice. Despite the numerous research conducted on antimicrobial efficacy on zebrafish, knowledge gaps loom large in assessing the efficacy of silica nanoparticles (SiNP) and the route of administration of domperidone (DP) (intramuscular).[12] There is a lack of evidence in identifying the properties of SiNPs, such as gene expression, enzymes, metabolites, effects on zebrafish, and accumulation patterns of nanoparticles in different organs and tissues.[13] Internal organs are infiltrated by smaller nanoparticles, whereas superficial parts, such as the gut, gills, and skin, are accumulated with larger nanoparticles.[14] The neurotoxic effects of SiNPs, such as apoptosis, axonal integrity, and gene expression, on embryos of zebrafish have been studied.[15]

As we venture deeper into the intricacies of zebrafish embryonic development, we cast a spotlight on these gaps in knowledge. In the quest for answers, this research aims to analyze the interactions among strives to contribute to our understanding of the intricate interactions among TAP, NP-TAP, and zebrafish embryonic development.

MATERIALS AND METHODS

Synthesis of nanosilica

A sterile conical flask was used to mix 1.57 mL of ammonia and 37 mL of ethanol as solvents, to which 5 mL of water was added gently. Upon continuous stirring for 5 min, 3 mL of TEOS was added, and the stirring was continued for the next hour.

Subsequently, the SiNPs were separated by the process of centrifugation at 10,000 rpm for 30 min. The resulting pellet was then dried using 60°C in a hot air oven.

Preparation of SiNPs based antibiotic combinations

A combination of medicaments, such as doxycycline, Flagyl, and ciprofloxacin were added to antibiotics with a composition of 100 mg each at a ratio of 1:1. Subsequently, these combinations were dissolved in 1 mL of distilled water and subjected to mixing on a vortex mixer for a duration of 10–15 min. This procedure was conducted to prepare the nano-silica-based antibiotic combinations.

Exposure of zebrafish embryos to tap and Np-TAP

Wild-type zebrafish (*D. rerio*) were recruited and preserved in separate tanks under controlled environmental conditions at a temperature of 28.2°C, a light/dark cycle of 14:10 h, with a pH of 6.8–8.5. The zebrafish were fed with dried bloodworms or given an appropriate diet twice every day. Each breeding tank consisted of a female and three male zebrafishes to produce embryos. Upon fertilization, viable eggs were collected and washed with a freshly prepared E3 media (without methylene blue) and distributed into 6-well, 12-well, and 24-well culture plates, with 20 embryos per well in 2 mL of solution.

For the experiments, E3 medium was prepared and added with a freshly prepared stock solution of TAP or Np-TAP at concentrations that include 1:10, 1:20, 1:40, 1:80, and 1:160. The solution was sonicated for 15 min to ensure even dispersion of nanoparticles while maintaining a pH of 7.2–7.3. Healthy fertilized embryos were exposed to these concentrations of TAP or Np-TAP for 24–96 h post-fertilization. Control groups were also established. Any non-viable embryos were removed from the exposed groups every 12 h, and all plates were covered and maintained at 28°C.

Evaluation of zebrafish embryos

Monitoring of embryonic development of Zebrafish was made under a stereo microscope during both the periods of exposure and subsequent fertilization. The concentrations

(1:10, 1:20, 1:40, 1:80, and 1:160) of TAP and Np- TAP were applied to the embryos from 24 to 78 h post-fertilization. The study endpoints included assessing embryonic mortality and hatching rates at 24-h intervals. Malformations observed in embryos and larvae in both the control and treatment groups were documented using a light microscope. Photographs of abnormal embryos were taken, and the percentage of affected embryos was recorded every 24 h.

RESULTS

The rate of hatching of embryos upon exposure to Np-TAP and the control group at concentrations of 10 μL, 20 μL, 40 μL, 80 μL, and 160 μL, were examined thoroughly over a 4-day period. Hatching was not seen on day 1 across groups, but a marked variation was evident on day 2. On Day 1, 30% hatching rates were observed with concentrations of 10 μL and 20 μL; however, 25% hatching rates with 40 μL, 20% and 15% with 80 μL and 160 μL, respectively [Figure 1].

On Day 2, complete hatching was observed across all the concentrations. It is evident that the Np-TAP's hatching is concentration-dependent, with varied hatching rates at different concentrations on Day 1 that turned into normal hatching eventually as the day progressed.

Notably, by the end of the 2nd day, all concentrations, including the control, showed complete hatching. The control group maintained a consistent 100% hatching rate throughout the 2 days.

Similarly, the viability rates upon exposure to Np-TAP and the control group at concentrations of 10 μL, 20 μL, 40 μL, 80 μL, and 160 μL were examined thoroughly over a 4-day period. The viability rates remained consistent across concentration and days in the control group, but the experimental group showed a concentration-dependent trend. Variations were not seen on day 1 across groups, but a marked variation was evident on day 2, with 80% viability rate of embryos to 80 μL and 70% rate with 160 μL concentration [Figure 2]. This trend is downward and it continued to reduce as the days moved further with a reduction in viability rates at higher concentrations on days 3 and 4. This implies that viability rates are also concentration dependent, similar to hatching rates.

The comprehensive analysis of the hatching rates of zebrafish embryos exposed to TAP at varying concentrations, including 10 μL, 20 μL, 40 μL, 80 μL, and 160 μL, alongside a control group were performed. Initially, none of the concentrations exhibited hatching, but 40% hatching rates were observed at lower concentrations and 25% was observed at the highest concentration of 160 μL. However, all the concentrations except for 80 μL and 160 μL, [Figure 3] exhibited complete hatching on the 2nd day, which shows a time-dependent response.

On day 4, no hatching was observed with 80 μL and 160 μL concentrations in contrast to the control group which exhibited complete hatching. This shows that the TAP also exhibits concentration-dependent trend with varied hatching rates at different concentrations on day 1 and 2, which was followed by no hatching at end of day 4. A comprehensive analysis of the viability rates of zebrafish embryos experimented with TAP at different concentrations showed full viability during the initial stage, but viability reduced substantially on the 2nd day when the embryos were exposed to higher concentrations and significant decline on the 4th day with highest concentration [Figure 4]. These show that TAP is also concentration-dependent.

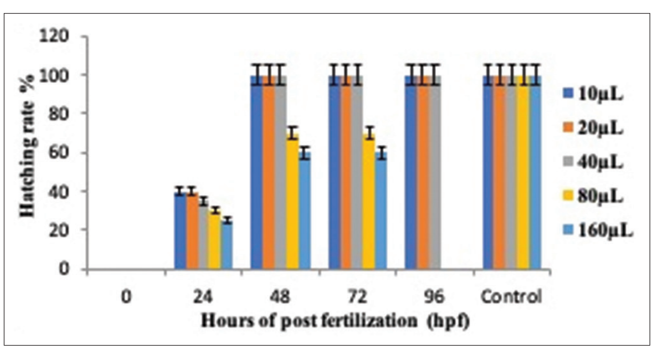

Figure 1: Hatching rate of zebrafish embryos treated with nanosilica based triple antibiotic paste

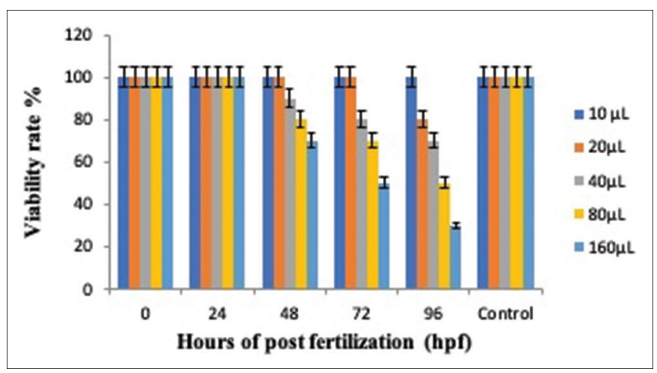

Figure 2: Viability rate of zebrafish embryos treated with nanosilica based triple antibiotic paste

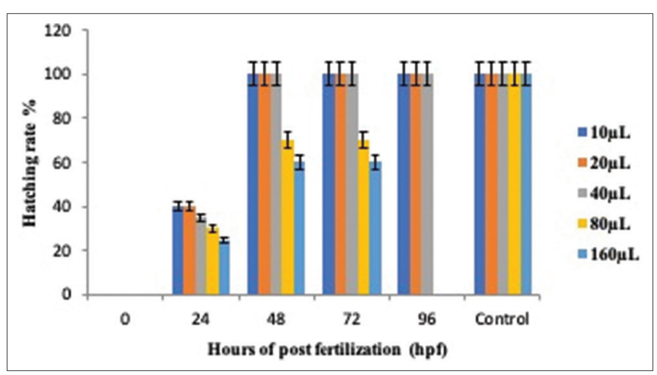

Figure 3: Hatching rate of zebrafish embryos treated with triple antibiotic paste

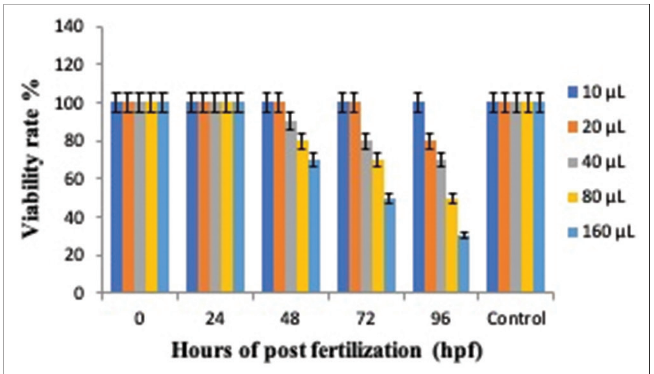

Figure 4: Viability rate of zebrafish embryos treated with triple antibiotic paste

DISCUSSION

The study analyzed the hatching rates and viability rates of zebrafish embryos at different concentrations for a 4 days period, which revealed interesting patterns in the hatching rates of zebrafish embryos when exposed to TAP and Np-TAP. At the initial stages, both TAP and Np-TAP exposure led to delayed hatching, particularly at higher concentrations of 160 µL. Highest concentrations of TAP and Np-TAP showed a decline in hatching on the 2nd day, which gradually changed and complete hatching occurred by the end of the 2nd day across concentrations. As the day progressed to the fourth, no hatching was noticed at the highest concentration in both TAP and Np-TAP groups; however, 100% hatching was observed in the control group. This shows that the hatching rates vary not only based on the duration, but especially the concentration. In specific, the hatching rates declined the most at the highest concentration in both TAP and Np-TAP groups, which shows the disruption of embryonic development in zebrafish. This result is in line with the findings observed in studies investigating the efficacy of TAP and Np-TAP as intracanal medicaments, which showed that they effectively eliminate pathogenic bacteria, making it highly beneficial for managing the polymicrobial nature of root canal infections.[12,13]

Similarly, the viability rates showed complete viability in the initial days with a substantial decline as the days progressed and the 4th day exhibited the most significant decline. The decline was more pronounced only to higher concentrations of TAP and Np-TAP. This finding is similar to hatching rates, and it is evident that both hatching and viability rates are concentration dependent; significant decline in hatching and viability rates were evident at higher concentrations on the 4th day.

The findings of the present study correlate with the previous studies where the impact of SiNPs were tested on zebrafish embryonic development, which demonstrated that combining DP with SiNPs reduced the toxicity of both DP and SiNPs, scaffolding ways to limit the cardiotoxic effects of DP.[14] Another study explored the effects of nano-SiO_2, a type of SiNP, on heart and blood vessel development in zebrafish, revealing cell apoptosis and increased endoplasmic reticulum stress.[15]

Another study investigated the effect of alumina nanoparticles (AlNPs) on neurodevelopmental toxicity in zebrafish larvae with respect to the size of the particle and found that smaller AlNPs showed malformations, genetic alterations, such as autophagy and oxidative stress.[16] The combination of SiNPs and cadmium on the cardiovascular system were experimented in a study, which identified the detrimental effects, such as heart rate and vascular endothelial cell damage getting intensified compared to the individual exposure.[17] On the other hand, nanosilica promotes dentine-pulp complex regeneration and achieved superior infection control in a few studies.[18,19] These studies make it evident that the SiNPs exhibit toxicological effects on zebrafish embryonic development, whereas the incorporation of nanosilica could improve the pharmacokinetics of TAP and its therapeutic index when introduced as an intracanal medicament.

The study's findings have several important implications, such as the importance of potential risks to the aquatic ecosystems, especially zebrafish and larvae, through the release of TAP and Np-TAP, as both materials are very commonly used in various industries and research laboratories. The study also demonstrated the concentration-dependent effects of TAP and Np-TAP on the zebrafish as the effect of hatching and viability vary based on the concentration, time, and size of the particles.[20] which emphasizes the importance of precision in dosage and controlling the experimental and environmental settings.[21]

Finally, this research contributes to the controversies raised on the usage of nanomaterials due to their potential ecological consequences. Nanosilica-based materials are used by various researchers and industries, although their compatibility and potential hazards have to be investigated through more clinical trials to conclude about their safety.[22] Overall, this study sheds light on the impact of TAP and Np-TAP on the embryonic development of zebrafish, providing valuable insights into their potential ecological consequences.

CONCLUSION

The effects of TAP and Np-TAP on the embryonic development stages of zebrafish are concentration-dependent. While the research highlighted some initial delays in hatching rates and a concentration-dependent impact on viability, it also indicates the potential for recovery and normalization of embryonic development over time. These findings underscore the adaptability of zebrafish in response to environmental stressors. It is considered that the study contributes to our growing understanding of the ecological implications of nanomaterials, encouraging future research to harness the benefits of these materials while mitigating potential environmental risks.

ETHICAL POLICY AND INSTITUTIONAL REVIEW BOARD STATEMENT

The study has been approved by the scientific review board with the reference number SRB/SDC/PhD/ENDO-1901/22/095.

PATIENT DECLARATION OF CONSENT

Not applicable.

DATA AVAILABILITY STATEMENT

Data are available in the article; further details can be requested by mail to the corresponding author.

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