

Cytotoxicity Evaluation of Zinc Oxide Nanoparticle-Coated Gutta Percha in Zebrafish: Optimal Concentrations for Enhanced Antibacterial Effect and Reduced Toxicity

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KEYWORDS

Root Canal treatment, Gutta Percha, Modified Gutta Percha, Antibacterial activity, Zebra fish

ABSTRACT

Background: Microorganisms have frequently been the cause of infections in the periapical tissue and dental pulp. The infected pulp tissue is removed and the root canal space is then prepared, cleaned and filled with a core root filling material. A persistent or secondary root canal system infection may be the cause of endodontic treatment failure. Concerning the issue, it is the need of the hour to look for alternative coating materials for Gutta Percha (GP) cones to increase their antimicrobial and antibiofilm efficacy for a successful outcome of the root canal therapies.

Aim: The aim of this study was to assess the cytotoxicity of commercial GP cones modified with thin deposition of zinc oxide nanoparticles.

Materials and Methods: Zinc oxide nanoparticles are extracted from zinc acetate and sodium hydroxide. Zinc oxide nanoparticles incorporated in PVA polymer. 1%, 3%, 5% zinc oxide nanoparticles are incorporated in PVA polymer separately. The surface of GP cones were modified by dip coating in PVA polymer. Group A- uncoated GP, Group B - coated GP. Cytotoxicity tests on zebrafish models were performed and 1%, 3%, 5% were compared.

Result: 1% Nanostructured zinc oxide coated GP shows less toxicity than 3%, 5% zinc oxide coated GP.

1. Introduction

Model organisms such as dogs, chimpanzees, pigs, rabbits, mice, rats, birds, Drosophila (fruit flies), Arabidopsis, E.coli and zebrafish are used in biomedical and toxicological research to identify and investigate biological phenomena [1]. Zebrafish have gained popularity recently as a vertebrate model for biological research. Zebrafish are commonly used as model organisms in biomedical research because of their rapid reproductive cycle and the transparency of their embryos which allows for easy observation [2]. The in vivo model using the Zebrafish (Danio rerio) and humans have similarities in genes homologous, genome, anatomy, physiological, cardiovascular, nervous, and digestive systems [3]. Dental materials can be tested for toxicity using zebrafish to identify any possible negative consequences such as neurotoxicity, tissue damage or developmental abnormalities [4–6]. It facilitates comprehension of the underlying pathways of toxicity and can direct dental material development to improve biocompatibility and safety [3,7]. And also Zebrafish embryos provide a unique chance to study the effects of nanoparticles on intact cellular systems that coordinate early embryonic development events [8,9].

In root canal therapy (RCT), the inflamed or infected pulp tissue is removed, the root canal space is prepared and cleaned, then Gutta Percha (GP), a rubber-based filling material, is filled with root canal cements. Because the therapeutic result of endodontic therapy depends on sealing the root canal space throughout the root canal, the final obturation step is an essential step. About 20% of GP cones are made up of GP (matrix), 66% are filled with zinc oxide, 11% are radiopacifiers with heavy metal sulfates and 3% are made up of waxes and/or resins. Endodontic therapy may not be successful if the

root canal system is infected either persistently or thereafter. Specifically, the very resilient intra-canal pathogen *Enterococcus faecalis* is frequently isolated in endodontic failure[10][11]. GP cones are frequently immersed in a variety of disinfectants to provide antibacterial action and enable material cleansing. It might change the physical properties of GP such as elasticity, tensile strength and elongation rate which could have an impact on the outcome of root canal treatment [12][13]. The present work proposes a novel approach to increase the antibacterial efficacy of GP by modifying the GP surface with deposition of a thin film of Zinc oxide nanoparticles(ZnONPs).

According to Alves Mj et al, Compared to the control, GP coated with ZnONPs film showed increased antibacterial activity. The antibacterial activity is explained by the electrostatic interaction between positively charged ZnO and negatively charged bacterial cell surface as well as by chemical and physical interfacial mechanisms [11]. Significant microbial toxicity is produced by the interfacial interactions, which cause bacterial cell wall destruction, increased permeability, increased intracellular oxidative stress and uptake of Zn²⁺ ions among other responses[14,15]. This study aimed to assess the toxicity of GP coated with ZnONPs by examining its effects on zebrafish embryos' heart rate, survival rate, hatching rate, development and comparing it to conventional GP.

2. Materials and Method

2.1 Coating Gutta Percha with zinc oxide nanoparticle

Pure ZnONPs are extracted for coating GP cones. Zinc acetate and sodium hydroxide which has pH of 8,11 are mixed and stirred for 30 minutes. The mixture was dried in the hot air oven at 80 degree celsius for 5 hours followed by calcination at 400 degree celsius for 1 hour. The resultant product is the pure ZnONPs. Pure ZnONPs (1%, 3%, 5%) were incorporated in PVA-polyvinyl acetate polymer separately which acted as a medium to coat GP surface. GP cones (Size 40, Dentsply Maillefer, Switzerland) from freshly opened boxes were organized into two groups(A-Uncoated GP, B-Coated GP). GP cones are coated with ZnONPs by dipping in PVA polymer which is incorporated with ZnONPs. Dip coating helps to get 3 dimensional coating of GP cones with thin zinc oxide film.

2.2 Maintenance of zebrafish

Zebra fish (*Danio rerio*) was used as a model organism for testing cytotoxicity. Zebra fish exhibit an exceptional set of characteristics such as small size, rapid development, embryonic transparency and potential to mimic human genes[16,17]. Adult zebrafish (wildtype -ABstrain, 4 months old) were obtained from Tarun Fish Farm (Chennai, India). Male and female fishes were separated and maintained in a 10 L glass tank at 28.5 degree celsius, with a 14/10 h light dark cycle. They were fed thrice daily with live brine shrimp (*Artemia salina*).The fishes were acclimatized for a period of 1 month, later the fishes were utilized for breeding and embryos were collected. The collected embryos were further analyzed under a microscope. Collected embryos underwent microscopic analysis, discarding unfertilized ones and transferring fertilized embryos to a six well plate and incubated in sterile water for 48 hrs.

2.3 Developmental toxicity in embryos

Zebrafish embryos are transparent during the early stages of development, enabling researchers to directly observe the formation of organs, tissues, and cellular processes without the need for invasive procedures. Within this investigation, the compounds employed to evaluate cytotoxicity of commercial GP(control) and ZnONPs coated GP with concentrations of 1%, 3%, 5%. The zebrafish subjects were exposed to the chemical agents for analysis by immersing them in solutions containing these substances for a set period of 24 hours. After the exposure, the embryos' developmental stages were examined under an inverted microscope to detect and assess any potential malformations.

2.6 Survival rate in Zebrafish embryos

Evaluating the survival rate of zebrafish embryos is a common method used to assess the effects of various chemicals and environmental factors on their development and overall viability. Zebrafish embryos are considered a valuable model organism due to their small size, transparency, and rapid developmental processes. Surviving embryos are counted on a daily basis, and any dead embryos are quickly removed. The survival rate (%) is assessed by monitoring key indicators such as egg coagulation, heart rate, and the presence or absence of spontaneous movement. Additionally, researchers may also examine the morphology, pigmentation, and organ development of the embryos and larvae to further evaluate the impact of the chemicals on their overall health and growth.

3. Results

3.1 Developmental toxicity in embryos

In this study, zebrafish larvae were typically exposed to the chemical agents by immersing them in solutions containing ZnONPs coated GP (1%, 3%, 5%) for a specified duration of 24 hours. The control group was Uncoated GP. The zebrafish larvae exhibited more developmental toxicity when exposed to 5% of ZnONPs coated GP than 1%, 3% of Coated GP and Uncoated GP. 3% ZnONPs coated GP also showed developmental toxicity but less than 5%. This resulted in the deformation of the zebrafish embryo, leading to the manifestation of defective zebrafish larvae. The alterations observed in the larvae following exposure to Coated GP are visually depicted in Figure 1.

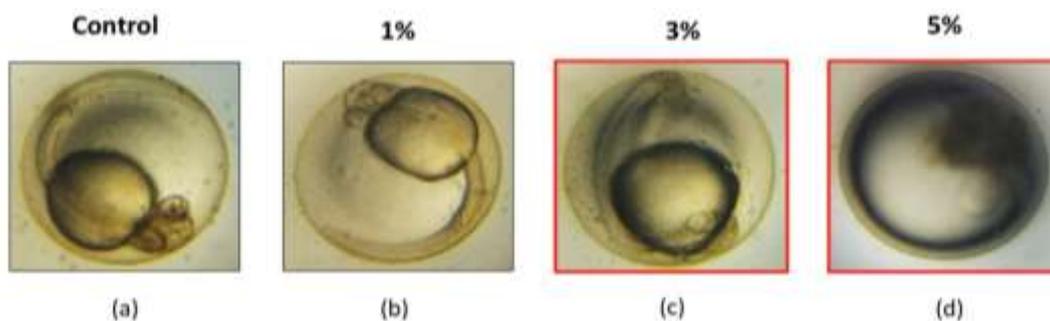


Fig 1: Developmental toxicity in embryos. (a)- Uncoated GP. (b),(c),(d)- 1%, 3%, 5% ZnONPs coated GP.

3.2 Heart rate in zebrafish embryos

The embryonic heart rate was recorded for one minute under the microscope and the average heart rate per minute was subsequently documented. The 1% ZnONPs coated GP did not yield a significant impact on the heart rates of zebrafish embryos compared to the control group. Elevated concentrations of coating 3%, 5% resulted in observable changes in heart rates, as illustrated in Figure 2.

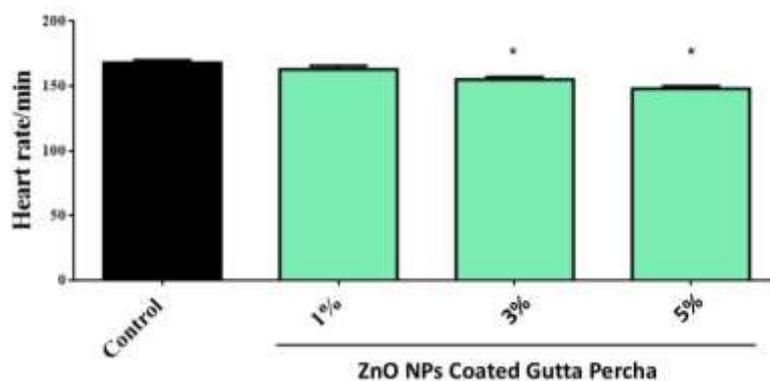


Fig 2: The heart rate of zebrafish embryos was investigated after being exposed to 4 different groups for 24 hours.

3.3 Hatching rate in zebrafish embryos

The hatching rate of zebrafish refers to the percentage of fertilized eggs that successfully hatch into larvae under specified conditions. The hatching rate can vary depending on several factors such as genetics, environmental conditions, embryo quality, incubation duration. Changes in the environment of zebrafish can indeed affect their hatching rate[18]. Notably, while 3%, 5%, ZnONPs coated GP showcased a significant reduction in hatching rates, 1 % ZnONPs coated GP demonstrated no significant impact on the hatching rates of zebrafish embryos when compared to the control group.

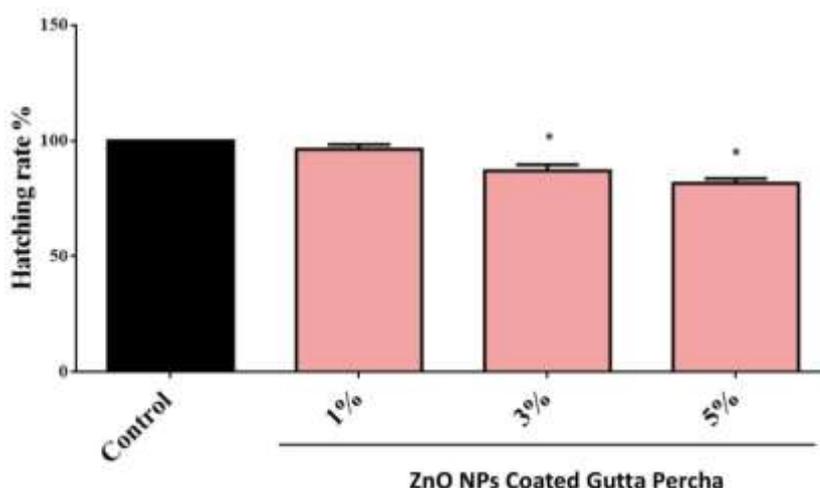


Fig 3: The hatching rate of zebrafish embryos was investigated after being exposed to 4 different groups for 24 hours.

3.4 Survival rate in zebrafish embryos

The survival rate of zebrafish is crucial for assessing the impact of environmental changes, chemicals, drugs and genetic manipulations. It serves as a quantitative measure of their resilience, adaptability, their ability to thrive or survive under experimental conditions, offering insights into biological processes, environmental impacts, and the evaluation of potential therapeutic interventions. Notably, 1% ZnONPs displayed higher survival rates compared to higher concentrations.

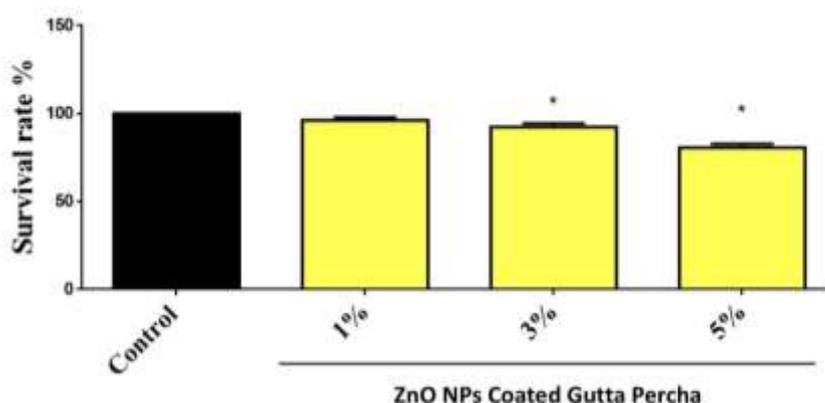


Fig 4: The Survival rate of zebrafish embryos was investigated after being exposed to 4 different groups for 24 hours.

4. Discussion

An ideal endodontic filling material should be biocompatible, ensuring it is well tolerated and does not interfere with or delay the healing process[19]. GP is extensively utilized because it is well accepted by the tissues of the host. GP has certain antibacterial qualities. Zinc oxide (ZnO), which is the dynamic material, is hydrolyzed to produce zinc particles (Zn²⁺)[20]. The materials inserted into the

root canal system might diffuse into the dentinal tubules. Moreover, the apical foramen or auxiliary root canals may be the route by which these materials come into touch with periodontal tissues. In this situation, a substance's toxicity is its biocompatibility with the surrounding tissues is crucial [21]. Diverse compounds, including bioactive glass, chitosan, nano propolis, nanocurcumin, silver nanoparticles, tetracycline and iodoform have been added to GP to boost its antibacterial capabilities in various investigations[19]. In this study, ZnONPs are used for surface modification of GP to increase its antibacterial properties.

For the assessment of in vivo nanotoxicity, small, inexpensive and sophisticated models are therefore particularly desirable[22]. Zebrafish embryos were exposed to different concentrations of ZnONPs coated GP to evaluate toxicity. A clear relationship exists between the survival rate of zebrafish embryos and the degree of toxicity resulting from varying doses of nanoparticles[23,24]. Since their introduction, the use of nanoparticles in endodontics has steadily increased due to the remarkable advantages and mechanisms of action that they have demonstrated[4,5]. A previous study revealed that the interaction of ZnONPs with CHX and Ca(OH)₂ improved dentin remineralization and had higher activity against *E. faecalis*. Furthermore, GP coated with ZnONPs worked well as sealants to inhibit the growth of *Escherichia coli* and *S. aureus*, lowering the possibility of reinfection following therapy[4,25].

The successful development of the embryo into larvae, which occurs between 48 and 72 HPF is shown by hatching rates. Zebrafish life cycles depend on hatching, which is connected to a series of biochemical and physical processes[26]. In toxicity assessments, the heart rate of zebrafish is often monitored as an indicator of the organism's physiological response to toxic substances. Zebrafish are commonly used in such studies due to their transparent embryos and well-documented genetics, making them a valuable model for understanding the effects of chemicals on cardiovascular function. The normal resting heart rate of zebrafish larvae is typically between 120 and 180 beats per minute (bpm)[27,28]. When exposed to toxic substances, changes in this baseline heart rate can indicate stress or adverse effects[29,30]. In healthy conditions, zebrafish embryos typically hatch within 24-48 hours post-fertilization. The hatching rate is usually high, often exceeding 90% in controlled laboratory settings. Exposure to toxic substances can adversely affect hatching[27].

In this work, GP's antibacterial activity is increased by coating it with zinc oxide nanoparticles. Although GP is known to be biocompatible, surface modified GP must first be tested for toxicity before entering clinical trials. Higher concentrations of ZnONPs coated GP showed developmental toxicity, decreased heart rate, hatching rate and survival rate in all evaluations. Less toxicity was seen in GP coated with 1% and 3% ZnONPs compared to GP coated with 5% ZnONPs. Endodontic failure can occur due to persistent infection or because of contaminated GP. Microorganisms often proliferate when there is improper handling and storage of materials[31]. According to de Almeida et al, ZnONs treatment significantly reduced the structure of a 7-day-old biofilm[32]. ZnONPs binding to the bacterial cell may have changed the permeability of the cell wall which allows proteins and other components to leak out which ultimately causes cell death[33,34]. Additionally, it greatly increased the dentinal tubule penetration depth[35]. Routine irrigation techniques are unable to eradicate microorganisms found in deeper zones of dentinal tubules. However, the microorganism that survived irrigation and mechanical instrumentation can be eliminated by altering the GP's surface with zinc oxide nanoparticles.

The limitation of this study is that the coating of GP with zinc oxide nanoparticles might affect the physical properties of the GP such as its flexibility, sealing ability or ease of manipulation. Ensuring that the coated material retains all the desirable properties of GP is crucial. It is also challenging to expose zebrafish to a coated GP environment. Future studies could focus on optimizing the formulation to enhance biocompatibility and reduce any potential adverse effects, ensuring that the material is safe for long-term use.

5. Conclusion

The toxicity test on zebrafish revealed that GP coated with 1% and 3% ZnONPs exhibited lower levels of toxicity compared to GP coated with 5% ZnONPs. These findings suggest that lower concentrations of ZnONPs on GP are less harmful to the zebrafish model, while still maintaining effective antimicrobial properties. This result also indicates that optimizing the concentration of ZnONPs can enhance the balance between minimizing toxicity and maximizing antibacterial efficacy. Future formulations of zinc oxide nanoparticle-coated GP can leverage these insights to achieve an effective antibacterial effect against microorganisms in the root canal, while ensuring safety and biocompatibility.

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