ORIGINAL RESEARCH

Embryonic Toxicology Evaluation of Ginger- and Clove-mediated Titanium Oxide Nanoparticles-based Dental Varnish with Zebrafish

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Abstract

Aim: The aim of the study is to evaluate the embryonic toxicology of ginger- and clove-mediated titanium oxide (TiO_2) nanoparticles (NPs)-based dental varnish with zebrafish (*Danio rerio*).

Materials and methods: Dental varnish was formulated using ginger, clove extract, and titanium dioxide NPs followed by the introduction of this test solution at concentrations of 1, 2, 4, 8, and 16 µL along with a control group with medium zebrafish embryos into a 6-well culture plate. After 2 hours of incubation, the embryos of zebrafish were tested and analyzed for hatchability and mortality rate using one-way ANOVA and *post hoc* Tukey's tests using statistical package for the social sciences (SPSS) software.

Results: The hatching rate of zebrafish embryos was greatest at 1 µL in a declining order when compared to the control group, whereas the mortality rate was greatest at 16 µL compared to the control group. On intergroup comparisons, one-way analysis of variance (ANOVA) has revealed a significance ($p = 0.00$) between the concentrations and testing parameters such as hatchability and mortality.

Conclusion: Within the limitations of the study, the zebrafish embryos exposed acutely to TiO_2 NPs at experimental doses have shown significant changes in their rate of deformity and capacity to hatch at 16- and 1-µL concentrations of the dental varnish formulation, respectively. Furthermore, studies are required to prove the efficacy of the formulation.

Clinical significance: Research and development of new formulations of various dental products is an ongoing process. One such segment is dental varnishes, wherein herbal resources and NPs mediated for improved efficacy against dental caries is an emerging alternative aiming to counteract the limitations posed by the traditional agents. To develop a new formulation of dental varnish, which is herbal resourced and NPs mediated, for an improved efficacy against dental caries.

Keywords: Acute toxicity, Antimicrobial activity, Antioxidant activity, Clove, Dental varnish, Ginger, Nanoparticle, Nitric oxide radical inhibition assay, Titanium oxide, Zebrafish.

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Introduction

The National Institute of Occupational Safety and Health, the American Society of Testing and Materials, and the International Organization for Standardization generally describe NPs as fibers or particles.[1] Nanoparticles are being synthesized increasingly often for *in vivo* applications, such as targeted medication delivery and diagnostics, where they have great potential.[2] To evaluate a given NPs' safety in a clinical or environmental situation, it is critical to understand how it interacts with cells and cell systems.[3]

Recent innovations have made it possible to produce multifunctional NPs, such as crystalline materials, fullerenes.[4] Nanoparticles are now used in a wide variety of scientific and non-scientific fields. They are also increasingly being used in production as chemically inert additives for things such as fillers, pigments, and anticaking agents. Due to their ability to target proteins or cells and their general ease of accessibility throughout the body, NPs are greatly useful in the field of bioimaging, medication administration, tissue engineering, and therapeutic treatments.[5]

Due to the demand for non-toxic NPs for the purpose of biomedical applications, toxicity is one significant factor to be considered since the widespread use of NPs and the growing need for their production might expose the environment to hazardous compounds or their byproducts.[6] Cytotoxicity can be assessed using *in vitro* cell culture tests, simple higher vertebrate models such as rodents and primates, as well as complex higher vertebrate models like sea urchins and daphnia.[7] For cell-level toxicity and genotoxicity investigations, cell lines and simple species are helpful, but larger vertebrates are required to comprehend complicated physiological relationships.[8]

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Primate models have similar problems to rodent models, but to a greater extent. Due to their great size, rodent models are expensive, their embryonic growth is long and challenging, they require a lot of material for testing, and their use raises ethical questions.[9]

Therefore, small, affordable, yet sophisticated models are highly desirable for *in vivo* nanotoxicity assessment. The zebrafish is an appealing, practical, and cost-effective solution in this situation.[10] Zebrafish are being used increasingly frequently to examine the biocompatibility of NPs.[11] Moreover, TiO_2 NPs have also been shown to have a positive antibacterial impact as a result of their photocatalytic properties. These free radicals of oxides and peroxides react with a wide range of microorganisms and provide a strong antibacterial action.[12]

Furthermore, TiO_2 NPs are more efficacious against gram-negative bacteria (e.g., *Candida albicans* and *Escherichia coli*) due to their thin cellular walls, and are less successful against gram-positive bacteria (e.g., *Staphylococcus aureus* and *Enterococcus faecium*) with thicker cell walls. This implies that cell wall complexity also influences how effective TiO_2 NPs are at inhibiting microbes.

Recent studies have discovered that adding metals, such as silver, to TiO_2 NPs boosts their ability to fight bacteria by enhancing light absorption and altering their photocatalytic properties.[13]

Dental varnishes are straightforward to use and give active ingredients such as fluoride or chlorhexidine to the teeth in a secure way.[14] The primary benefit of varnish's anticaries activity is its local action at the tooth/plaque interface, where it encourages remineralization while lowering demineralization. Also, *Streptococcus mutans* is inhibited from generating acid.[15]

With the background of numerous studies conducted on synthesis and evaluation of various properties of NPs such as zirconium oxide, selenium, halloysite nanotubes, silymarin/hydroxyapatite, chitosan nanocomposites, herbal formulated silver NPs, nanoemulsion, and oleoresins by our colleagues,[16–28] the aim of this study is to evaluate the embryonic toxicology of ginger- and clove-mediated TiO_2 NPs based dental varnish with zebrafish.

Materials and Methods

Dental Varnish Preparation

To make dental varnish, 500 µL of NPs, 4 mL of ethanol, 0.9 mL of acetic acid, and 4.6 mL of diH_2O were combined to make a total of 10 L.

Evaluation of Acute Cytotoxicity Using Zebrafish Embryos

Zebrafish (10 females and 15 males) were purchased from suppliers in Chennai, Tamil Nadu, India and kept in separate tanks at a temperature of 28°C in a light-and-dark cycle of 14 and 10 hours, with a pH maintained between 6.8 and 8.5. Shrimp and dry flakes as food were given to the fish twice a day. A transparent block was used to manually separate the sexes for the whole night; it was then taken away for reproduction the following morning. One female fish was crossed with two male fish to produce the fish embryos. Viable eggs were retrieved and rinsed using an E3 medium consisting of 5 mmol/L sodium chloride, 0.17 mmol/L potassium chloride, 0.33 mmol/L calcium chloride, and 0.33 mmol/L magnesium sulfate with no added methylene blue and a pH maintained at 7.2. The fertilized eggs were incubated into 10 different 6-well culture plates keeping experimental and control groups separate with 3 mL of E3 medium and 100 mg/L of standard TiO_2 contrast solution. The control group consisted of only E3 medium and the zebrafish embryos. The sample size was determined as five experimental groups with NPs mediated dental varnish and one control group with five embryos and the medium in each well. Dental varnish at concentrations of 1, 2, 4, 8, and 16 µL was used to incubate the fertilized embryos for 24 hours post fertilization (hpf) to 96 hpf. Different concentrations of dental varnish were exposed to the experimental group containing 5 embryos for a period of 96 hours as the normal hatching period of the zebrafish embryo is from 48 hpf to 72 hpf.

The hatching rate and viability were recorded every 24 hours. In addition, 5 embryos were maintained as a control group. If any of the fishes were dead, they were recorded and removed. At periodic intervals (every 2 hours) during the exposure to the mouth rinse, the embryonic development of the zebrafish embryo was observed under a stereo microscope. The end point of the experiment was to assess the developmental toxicity including mortality, embryo hatching rate, and larva viability. The photographs of the developing embryos were taken using a stereomicroscope. The factors considered to evaluate the results were based on the capacity of the embryos to hatch, number of embryos that were hatched without any malformations, followed by their survival phase and mortality with the help of periodic photographs taken under stereomicroscope. The raw data were transferred in an Excel sheet and using SPSS software one-way analysis and *post hoc* Tukey's test analysis was performed.

Results

In this study, zebrafish embryos were successfully hatched at concentrations of 100 mg/L TiO_2 NPs. Figures 1 and 2 show the hatching rate and viability rate of zebrafish embryos against the newly prepared dental varnish. The rate of hatching was in a declining order with the greatest (78%) at 1 µL concentration followed by 2 µL (63%), 4 µL (52%), 8 µL (31%), 16 µL (25%), and control at 100%. In reciprocation to the values of the hatching rate, the percentage of viability was greater (82%) at the lowest concentrations of 1 µL, 2 µL (78%), 4 µL (73%), 8 µL (60%), and least (54%) at 16 µL when compared with the control group (100%). For hatchability one-way ANOVA and *post hoc* Tukey's test,

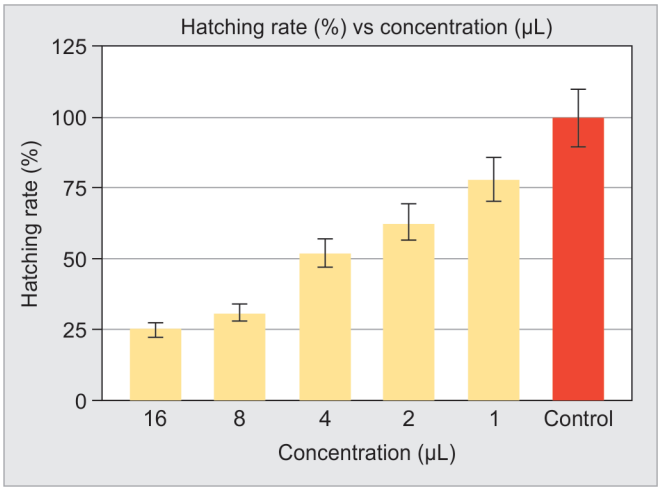

Fig. 1: Zebrafish study of clove and ginger-mediated TiO_2 NPs-based dental varnish at various concentrations from 1, 2, 4, 8, and 16 µL and the hatching rate with a control. The *x*-axis shows the concentration of the NPs and the *y*-axis shows the hatching rate

Tables 1 and 2 revealed significant ($p = 0.00$) variations in hatching delay between the groups at various concentrations. Whereas on comparing intergroup samples of various concentrations toward the mortality rate of the zebrafish embryos, Tables 3 and 4 refer to the mortality rate of the zebrafish embryos at various concentration with a significance of $p = 0.00$. Based on the above zebrafish embryonic development results, it was inferred that the most favorable concentration of the new formulation is 1 µL with the least mortality and highest hatching rate without any malformations. Malformations such as yolk sac and pericardial edema tail bent, spinal curvature, and axis bent were not detected during the observations. The embryos exposed in the control solution (E3 medium) did not show any developmental defects with a hatching and mortality rate of 100%.

Discussion

Due to the rapid development in technological aspects, nanomaterials are now extensively used in a variety of sectors/industries such as chemical, electronics, pharmaceuticals, cosmetics, and much more. As a significant one, TiO_2 NPs have been widely used as catalysts, coating substances, cosmetic fillers, nanoceramics, and other applications.[29]

The results of Vicario–Pares concur with this. The variation in TiO_2 NPs' crystal forms and particle sizes, which could have varying toxicity effects, could be the cause of the disparity in experimental results. Along with their physical and chemical characteristics, TiO_2 NPs' dispersion and precipitation rates in solution play a crucial role in determining how poisonous they are.[30] Earlier toxicology investigations were made in a systematic manner starting from *in vitro* followed by testing on animal models and later human trials. However, this causes a greater difficulty due to the availability of various cell lines at different concentrations. Due to the structural and biomechanical similarities of humans and zebrafish at the cellular level, currently, zebrafish is a popular vertebrate model for toxicity investigations as it is the fastest test model to analyze any genetic changes for early test regimens.[31] Hence to predict the chemical risk of new formulation toward humans, vertebrate toxicity studies such as zebrafish embryonic toxicity test was considered for this study. Short-term exposure to TiO_2 NPs caused no obvious harm to zebrafish embryos. TiO_2 NPs might be prevented from entering zebrafish embryos by the eggshell. TiO_2 NPs and intraembryonic cells do not directly interact when the system is growing. The embryo's dietary requirements are mostly met by the yolk sac.[30] As the findings mentioned in the above studies titanium dioxide NPs have proven to be of no harm to the zebrafish embryos, hence NPs were considered in this study for the evaluation.

Since external nutrients are not needed, they cannot be exchanged with other substances and have no observable impact on the growth of the embryo. The development of zebrafish embryos was not affected by TiO_2 NPs, however, it was discovered that extrinsic physicochemical variables, such as ultraviolet (UV) light, might increase TiO_2 NPs' acute toxicity.[32] Xiong et al. have also tested TiO_2 NPs for their chronic toxicity in adult zebrafish in addition to their acute toxicity to developing embryos. The enhancement and elimination of anatase and crystalline (gold red) TiO_2 NPs under long-term exposure scenarios were investigated using adult zebrafish. The results showed that prolonged subjection to TiO_2 NPs caused some enrichment in zebrafish, but there was no proof of bioaccumulation.[32]

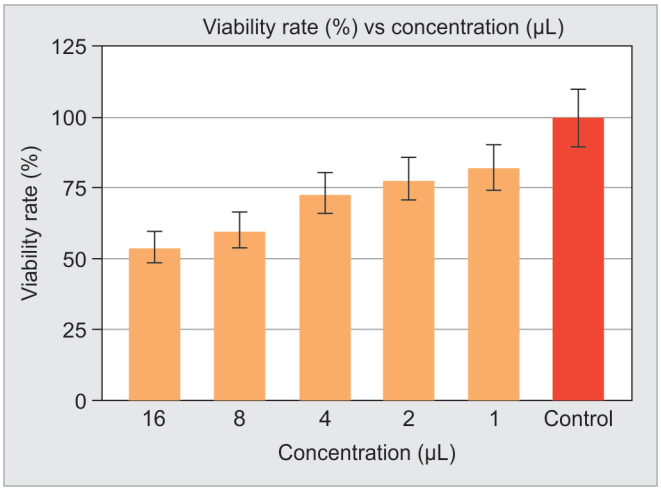

Fig. 2: Zebrafish study of clove and ginger-mediated TiO_2 NPs-based dental varnish at various concentrations from 1, 2, 4, 8, and 16 µL, and the viability rate with a control. The *x*-axis shows the NPs concentration and the *y*-axis exhibits the viability rate

Limitations

With an increase in the concentration of the NPs apart from hatching and mortality rate factors like exposure time, developmental abnormalities of zebrafish also should be studied to strengthen the results of the new formulation. Due to lack of flexibility with a moderate foresight and limitation to translate the values, zebrafish embryo toxicology test is still an acceptable intermediate test that needs further progressive evaluation.[31]

Conclusion

Within the limitations of the study, zebrafish embryos exposed acutely to TiO_2 NPs at experimental doses have detected changes in their capacity to hatch or their rate of mortality at varied concentrations of TiO_2 NPs. To further evaluate the biosynthesis of the formulation, characterization of NPs along with its biomedical applications such as antibacterial, antioxidant, and anti-inflammatory effects of the NPs should further be evaluated.

Table 1: One-way ANOVA performed at various concentrations of test solution in comparison with hatchability of zebrafish embryos

One-way ANOVA					
Hatching					
	Sum of squares	*df*	*Mean square*	*F*	*Significance*
Between groups	39893.333	5	7978.667	1133.811	0.000
Within groups	380.000	54	7.037		
Total	40273.333	59			

Table 2: *Post hoc* Tukey's tests performed between various concentrations toward their hatchability

Multiple comparisons							
Dependent variable: Hatching							
			Mean difference			*95% CI*	
	I groups	*J groups*	*(I – J)*	*SE*	*Significance*	*Lower bound*	*Upper bound*
Tukey's HSD test	1 µL	2 µL	15.000*	1.186	0.000	11.49	18.51
		4 µL	26.000*	1.186	0.000	22.49	29.51
		8 µL	46.000*	1.186	0.000	42.49	49.51
		16 µL	53.000*	1.186	0.000	49.49	56.51
		Control	–22.000*	1.186	0.000	–25.51	–18.49
	2 µL	1 µL	–15.000*	1.186	0.000	–18.51	–11.49
		4 µL	11.000*	1.186	0.000	7.49	14.51
		8 µL	31.000*	1.186	0.000	27.49	34.51
		16 µL	38.000*	1.186	0.000	34.49	41.51
		Control	–37.000*	1.186	0.000	–40.51	–33.49
	4 µL	1 µL	–26.000*	1.186	0.000	–29.51	–22.49
		2 µL	–11.000*	1.186	0.000	–14.51	–7.49
		8 µL	20.000*	1.186	0.000	16.49	23.51
		16 µL	27.000*	1.186	0.000	23.49	30.51
		Control	–48.000*	1.186	0.000	–51.51	–44.49
	8 µL	1 µL	–46.000*	1.186	0.000	–49.51	–42.49
		2 µL	–31.000*	1.186	0.000	–34.51	–27.49
		4 µL	–20.000*	1.186	0.000	–23.51	–16.49
		16 µL	7.000*	1.186	0.000	3.49	10.51
		Control	–68.000*	1.186	0.000	–71.51	–64.49
	16 µL	1 µL	–53.000*	1.186	0.000	–56.51	–-49.49
		2 µL	–38.000*	1.186	0.000	–41.51	–34.49
		4 µL	–27.000*	1.186	0.000	–30.51	–23.49
		8 µL	–7.000*	1.186	0.000	–10.51	–3.49
		Control	–75.000*	1.186	0.000	–78.51	–71.49
	Control	1 µL	22.000*	1.186	0.000	18.49	25.51
		2 µL	37.000*	1.186	0.000	33.49	40.51
		4 µL	48.000*	1.186	0.000	44.49	51.51
		8 µL	68.000*	1.186	0.000	64.49	71.51
		16 µL	75.000*	1.186	0.000	71.49	78.51

*Mean difference is significant at the 0.05 level. CI, confidence interval; HSD, honestly significant difference; SE, standard error

Table 3: One-way ANOVA performed at various concentrations of dental varnish in comparison with mortality of zebrafish embryos

One-way ANOVA					
Mortality					
	Sum of squares	*df*	*Mean square*	*F*	*Significance*
Between groups	13782.750	5	2756.550	447.142	0.000
Within groups	332.900	54	6.165		
Total	14115.650	59			

Table 4: *Post hoc* Tukey's tests performed between various concentrations toward the mortality rate of zebrafish embryos

Multiple comparisons							
Dependent variable: Mortality							
			Mean difference			*95% CI*	
	I groups	*J groups*	*(I – J)*	*SE*	*Significance*	*Lower bound*	*Upper bound*
Tukey's HSD test	1 µL	2 µL	4.000*	1.110	0.009	0.72	7.28
		4 µL	9.000*	1.110	0.000	5.72	12.28
		8 µL	22.900*	1.110	0.000	19.62	26.18
		16 µL	28.000*	1.110	0.000	24.72	31.28
		Control	–18.000*	1.110	0.000	–21.28	–14.72
	2 µL	1 µL	–4.000*	1.110	0.009	–7.28	–0.72
		4 µL	5.000*	1.110	0.000	1.72	8.28
		8 µL	18.900*	1.110	0.000	15.62	22.18
		16 µL	24.000*	1.110	0.000	20.72	27.28
		Control	–22.000*	1.110	0.000	–25.28	–18.72
	4 µL	1 µL	–9.000*	1.110	0.000	–12.28	–5.72
		2 µL	–5.000*	1.110	0.000	–8.28	–1.72
		8 µL	13.900*	1.110	0.000	10.62	17.18
		16 µL	19.000*	1.110	0.000	15.72	22.28
		Control	–27.000*	1.110	0.000	–30.28	–23.72
	8 µL	1 µL	–22.900*	1.110	0.000	–26.18	–19.62
		2 µL	–18.900*	1.110	0.000	–22.18	–15.62
		4 µL	–13.900*	1.110	0.000	–17.18	–10.62
		16 µL	5.100*	1.110	0.000	1.82	8.38
		Control	–40.900*	1.110	0.000	–44.18	–37.62
	16 µL	1 µL	–28.000*	1.110	0.000	–31.28	–24.72
		2 µL	–24.000*	1.110	0.000	–27.28	–20.72
		4 µL	–19.000*	1.110	0.000	–22.28	–15.72
		8 µL	–5.100*	1.110	0.000	–8.38	–1.82
		Control	-46.000*	1.110	0.000	–49.28	–42.72
	Control	1 µL	18.000*	1.110	0.000	14.72	21.28
		2 µL	22.000*	1.110	0.000	18.72	25.28
		4 µL	27.000*	1.110	0.000	23.72	30.28
		8 µL	40.900*	1.110	0.000	37.62	44.18
		16 µL	46.000*	1.110	0.000	42.72	49.28

*Mean difference is significant at the 0.05 level. CI, confidence interval; HSD, honestly significant difference; SE, standard error

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