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ORIGINAL ARTICLE

TOXICITY ASSESSMENT OF ZINC OXIDE NANOPARTICLES AND ORGANIC POLLUTANTS ON ZEBRAFISH USING BLISS INDEPENDENCE

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ABSTRACT

Background: Zinc oxide nanoparticles (ZnONPs) and chlorhexidine (CHX), a persistent organic pollutant (POP), are widely used engineered nanomaterials (ENMs) that may threaten freshwater ecosystems. While ZnONPs are considered safer than metal salts, they can still induce oxidative stress, membrane damage, and ion release. The co-occurrence of CHX and ZnONPs in aquatic systems raises concerns over combined toxic effects, which conventional risk assessment methods may not adequately address.

Aim: To evaluate the individual and combined acute toxicity of ZnONPs and CHX on zebrafish (*Danio rerio*) embryos, and to characterize their interaction using the Bliss Independence model.

Materials and Methods: Zebrafish embryos (<3 h post-fertilization) were exposed for 72 h to ZnONPs, CHX, and their binary mixtures (1:1) at IC_{20} and IC_{50} concentrations in 24-well plates under controlled conditions. ZnONPs were synthesized and characterized by UV–Vis spectroscopy and dynamic light scattering (DLS). Endpoints assessed included mortality, heart rate reduction, teratogenicity, and spinal deformities. Interaction effects were analyzed using the Bliss Independence model, with statistical evaluation by one-way ANOVA and Tukey's post hoc test.

Results: The 72-h LC_{50} values were 4.5 ppm (ZnONPs) and 3.9 ppm (CHX). Mixture toxicity varied with ratio and concentration: antagonism predominated at IC_{20} and IC_{50} (CI > 1) in 1:1 and 1:2 ratios, while CHX-rich mixtures showed synergism (CI < 1) at IC_{70}. CHX-heavy mixtures exhibited the highest lethality and teratogenicity, whereas ZnONP-rich mixtures reduced CHX toxicity, likely via decreased bioavailability or ROS scavenging.

Conclusion: Mixture toxicity of ZnONPs and CHX is ratio- and endpoint-dependent, highlighting the need for interaction-based ecological risk assessments.

Keywords: Zebrafish, Zinc oxide nanoparticles, Chlorhexidine, Ecotoxicology, Bliss Independence model, Antagonism, Synergism.

INTRODUCTION

The increased usage of engineered nanomaterials (ENMs) as well as persistent-able organic pollutants (POPs) in consumer goods and industrial applications has raised alarm bells for the environmental safety of fresh and marine water ecosystems. Of all the ENMs, zinc oxide nanoparticles (ZnONPs) are the most popular and commonly used material in sunscreens, cosmetics, paints, and antibacterial substances due to their high chemical reactivity, photocatalytic ability, and unique characteristics including high surface area[1]. But these characters also make ZnONPs as biologically active and capable of producing reactive oxygen species (ROS), that could damage cell membranes, by releasing zinc ions (Zn^{2+}). These ions are highly cytotoxic to aquatic organisms[2,3]. Besides, the existence of Zn, is considerably found as a compounded and or salted- form rather than as single-contaminants in nature. In compound forms, it is usually occurring with other organic pollutants. Among the organic compounds, the common pollutants such as chlorhexidine (CHX), bisphenol A (BPA), diethylhexyl phthalate (DEHP), and triclosan (TCS) are seemed to be highly reactive with ZnONPs. These organic compounds are easily found in aquatic systems due to their extensive uses as disinfectants, plastics, and personal care products. Particularly, the chlorhexidine becomes increasingly recognized as a significant

emerging contaminant in hospital and domestic effluents[4]. As combination, both ZnONPs and those organics together could severely affect the organisms due to their increased bioavailability and possible interactive effects with physiological levels[5].

Although regarded as less toxic than heavy metal salts, ZnONPs could cause several adverse effects from the chronic, sublethal exposure. That severity may range from oxidative stress, developmental abnormalities, immunotoxicity and or behavioral changes, to aquatic invertebrates[6]. Risk assessment on the ecosystem health threats from their persistent inputs to surface waters from industries and households should consider long-term, low-dose exposure, and mixture conditions because of the biomagnification[7]. As chlorhexidine (CHX) is recorded as a common disinfectant from hospital discharge and personal care products, it becomes an emerging contaminant in aquatic ecosystems[8-10].

Besides, the accumulation of the several such kinds of contaminants react with themselves and resulting in a complex contaminant mixture. Due to the compounded ones, these could act additively, synergistically, and or antagonistically at physiological functions of the organisms. Hence, risk prediction based on single-compound studies becomes complicated, and insufficient for calculating the risk assessment. In modern era, for assessing the toxicity of such nanoparticles, their co-contaminants such as organics should also evaluate for assessing the bioavailability, uptake, and activity to correlate the amplification or inhibition of their respective toxicity[11,12]. The Bliss Independence approach is one of the popular statistical methods for analyzing such interactions and making better ecological risk assessments. It predicts the joint effect of two substances from their single effects under the assumption that they act independently.

In aquatic toxicology, the zebrafish (*Danio rerio*) represent a well-known vertebrate model, valued for their fast developmental rate, clear embryos, and physiological similarity to higher vertebrates. Research into toxicity has shown that exposure to both ZnONPs and organic pollutants can cause developmental toxicity, oxidative stress, and behavioral impairment in zebrafish, thus indicating their susceptibility towards these pollutants[15]. A very important aspect of ecologically relevant risk assessment is gaining insight into possible interaction effects through assessment of mixture toxicities in fixed-ratio experiments in zebrafish, relying on Bliss modeling[16]. Differences from these predictions might then categorize interaction types as synergistic, antagonistic, or additive, which is particularly applicable to combinations like ZnONPs and chlorhexidine, which have particular modes of action[13,14].

This study contributes to the emerging field of mixture toxicology by providing knowledge on the interactions of ZnONPs with common environmental contaminants in an aquatic ecosystem.Through the integration of classical ecotoxicological endpoints with a mixture model approach, we aim to bridge the gap between laboratory investigation and natural exposure scenarios[3,5].

For this study, we focused on evaluating the toxicities of ZnONPs, both individually and also with the organic pollutant chlorhexidine, on zebrafish. Fixed-ratio mixture experiments were carried out for an excellent understanding of concentration-based lethal responses for 72 hours and using the Bliss Independence model to determine whether interactions between toxicants were synergistic, additive, or antagonistic[12,17,18]. The purpose of the study is to provide traditional ecotoxicological measures together with quantitative mixture modeling with a better understanding of pollutant interactions, thus bridging the gap between laboratory studies and the real field issues of aquatic life[11,17].

MATERIALS AND METHODS

Zebrafish embryos (lesser than 3h old) were used as the model aquatic model to assess ecotoxicological interactions of Zinc Oxide Nanoparticles (ZnONPs) with selected organic pollutant, i.e., chlorhexidine. The ZnONPs were synthesized and their size and surface charge were measured using UV–Vis spectroscopy and dynamic light scattering (DLS), respectively. The organic pollutants were purchased from USA Sigma-Aldrich and were freshly made up in dechlorinated water.

Zebrafish Acute Toxicity Assay

Embryos less than 3 hours old were exposed (OECD, test No. 236) in 24-well plates to individual compounds (0.001, 0.01, 0.05, and 0.5 ppm, respectively) and their binary combinations (1:1, 1:2, 1:3, 1:4, 2:1, 3:1, 4:1) at IC_{20}, IC_{50} and IC_{70} equivalent concentrations. Exposure was performed under controlled conditions (20 ± 1°C, 16:8 h light:dark) for 72 hours. Endpoints measured included Mortality (%), Heart Rate Reduction (%), Teratogenicity (%) and Spinal Cord Deformity (%). Three biological replicates were performed per group.

Combination Analysis Using Bliss Independence

To assess interaction between ZnONPs and pollutants, the Bliss Independence model was employed. The expected combined effect (E_AB,exp) was calculated using:

$$E\_AB = E\_A + E\_B - (E\_A \times E\_B)$$

Where, E_A and E_B are the individual effects and E_AB are the combined effects . The observed effect (E_AB,obs) was subtracted from E_AB,exp to calculate ΔE. Synergism was indicated when ΔE > 0, additivity at ΔE = 0, and antagonism when ΔE < 0. Mortality and sublethal endpoints were analyzed using Microsoft Excel and R-based synergy models.

Statistical Analysis

Results were shown as the mean ± Standard Deviation. Records were made in triplicate and then analyzed by one-way ANOVA with Tukey's posttest. Statistical significance was defined as $p < 0.05$ [19]. The LC_{50} IC_{20}, IC_{50}, and IC_{70}levels were calculated using non-linear regression. Graphs and interaction plots were performed for all levels of combination condition. Graphpad prism, ver. 8.2 was used for statistical analysis.

RESULTS

The present study evaluated the individual and combined toxicological effects of zinc nanoparticles (ZnONPs) and chlorhexidine on *zebra fish*. Acute embryo toxicity assay was conducted following OECD, test No. 236.guidelines, and combinatorial toxicity was assessed using the Bliss Independence model. The study investigated 72h responses at concentrations at different fixed ratios *i.e.*, 1:1, 1:2, 2:1, 3:1, 1:3, 1:4, 4:1 respectively. The Bliss Independence model allowed quantitative classification of interactions as synergistic, additive, or antagonistic.

3.1 Individual Compound Toxicity: ZnONPs and CHX:

The zebrafish embryos were treated with increased concentration of the ZnONPs and CHX (0.001, 0.01, 0.05, and 0.5 ppm) and displayed with concentration-dependent activity on (a) mortality (%),(b) teratogenicity (%),(c) hatching rate (%), and (d) heart beat reduction (%), respectively. CHX showed higher toxicity than ZnONPs attained halted developmental endpoints (IC_{20}, IC_{50} and IC_{70}) even at the lower concentrations. The IC_{50} values for them were 0.035 and 0.12 ppm, respectively, confirming a higher intrinsic toxicity of CHX. Dose- response curves of them showed a steeped slope for CHX, also revealing an immediate transition of the higher toxic capability from the sub- toxic concentrations. There was a highly significant effect (ANOVA, $p < 0.01$) on various responses at given concentrations, thereby supporting the dose-dependent effects and biological activity of each agent.

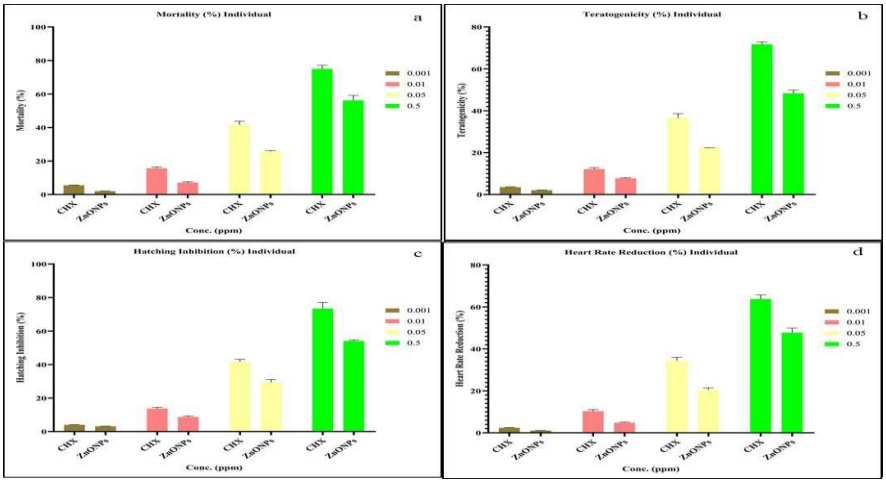

Figure 1. Concentration-dependent effects of Chlorhexidine (CHX) and Zinc Oxide Nanoparticles (ZnONPs) on zebrafish embryos, showing changes in (a) mortality (%),(b) teratogenicity (%),(c) hatching rate (%), and (d) heart rate reduction (%). Data are expressed as mean ± SD from triplicate experiments across tested concentrations (n=3)

3.2 Mortality outcomes at IC_{20} revealed differential combination toxicity profiles in zebrafish embryos treated with Chlorhexidine and Zinc Oxide Nanoparticles

At IC_{20}, mortality rates differed within different concentrations of the combined chemicals. The highest mortality for the tested combinations at this concentration was for the 1:4 ratio (ZnONPs:CHX), where a mortality of 37.7 ± 1.6% was recorded, showing that significant toxicity was induced by sublethal concentrations (Figure.2). The 1:1 and 1:2 ratios also produced moderately toxic effects, while concentrations dominated by ZnONPs, such as 2:1, 3:1, and 4:1,

showed a gradual decline in mortality. However, in all cases, the CI values at IC_{20} exceeded 2.0, with a CI of 2.89 for the 1:1 ratio and 2.08 for the 1:2 ratio, classifying the interaction as strongly antagonistic. It indicated that while CHX is still largely lethal, ZnONPs prevent interaction strength from reaching additive predicted levels, either by surface interactions or low bioavailability.

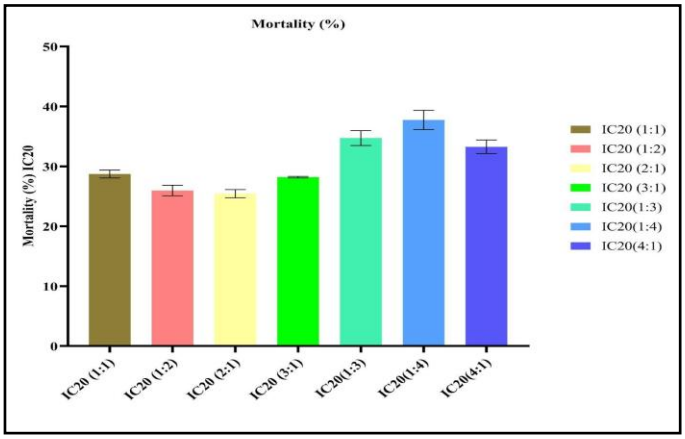

Figure 2. Mortality (%) in zebrafish embryos combination of Chlorhexidine (CHX) and Zinc Oxide Nanoparticles (ZnONPs) at IC_{20}, expressed as mean ± SD from three independent experiments.

3.3 At IC_{20}, Teratogenicity patterns indicated varied combination toxicity responses in zebrafish embryos treated with chlorhexidine and zinc oxide nanoparticles.

At IC_{20}, lesser degree of teratogenicity was evident, with Macroscopic developmental malformations being most pronounced in the mixtures rich in chlorhexidine. Ratios 1:2 and 1:3 exerted the highest teratogenic effects, showing symptoms that included yolk sac edema, truncated body axes, and improper dorsal curvature. These observations were contrasted by conditions of the 3:1 and 4:1 ratios with less concentration in ZnONPs, where the abnormalities were comparatively less (Figure.3). CI values indicated antagonism across all ratios, but the actual developmental harm in the CHX-rich groups infers that biological outcomes do not always match the calculated interaction models, particularly for sublethal endpoints.

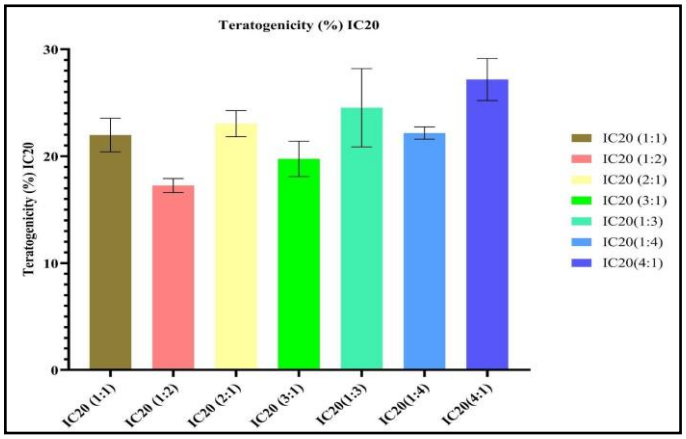

Figure 3. Teratogenicity (%) in zebrafish embryos combination of Chlorhexidine (CHX) and Zinc Oxide Nanoparticles (ZnONPs) at IC_{20}, expressed as mean ± SD from three independent experiments.

3.4 Combined effect CHX and ZnONPs on Heart Rate Reduction at IC_{20}

Heart rate suppression was evidenced even at IC_{20}, with combinations dominated by CHX, particularly 1:2 and 1:3, causing reductions of greater than 20% (Figure. 4). The 1:1 ratio yielded a more modest reduction (~15%), while ZnONPs-heavy ratios produced lesser effects. These results highlighted the sensitivity of the cardiac function in presence of CHX-induced stress. Interaction modeling suggested a strong antagonism; yet, cardiac system assessment revealed the functional impact of CHX toxicity even in antagonistic combinations with ZnONPs.

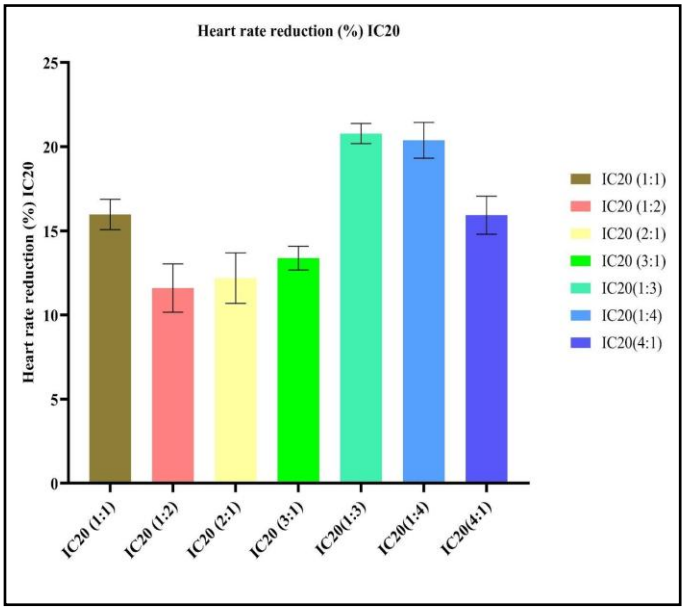

Figure 4. Combined effect CHX and ZnONPs on Heart Rate Reduction at IC_{20}

3.5 Combined effect CHX and ZnONPs on Spinal Cord Deformity at IC_{20}

Spinal deformities (mentioned as teratogenicity as in overall effects) at IC_{20} were relatively mild (Figure.5) but still present, with 1:4 and 1:3 ratios producing the highest deformity rates (~13-14%). These deformities, including axial misalignment and lateral curvature, were less frequent in ZnONPs-dominant mixtures, which also fit the trends seen in other endpoints. Overall, the degree of severity was low at IC_{20}; however, structural anomalies being there always reinforced the idea of CHX posing high sensitivity acting at an early stage of development, even in terms of antagonistic interaction conditions.

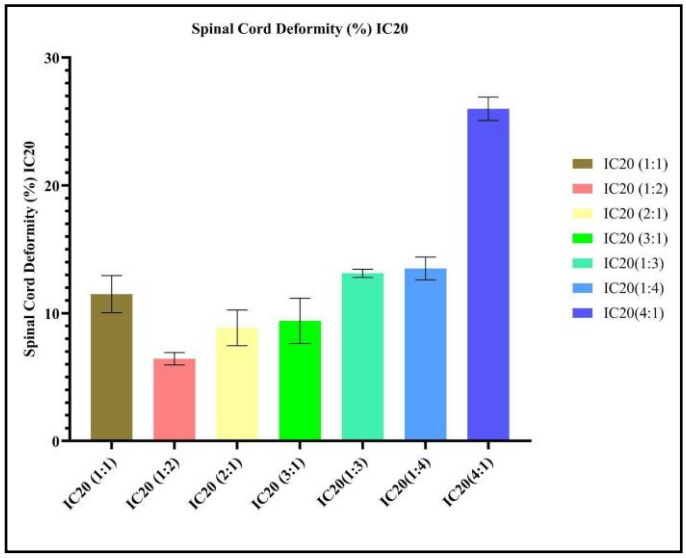

Figure 5. Combined effect CHX and ZnONPs on Spinal Cord Deformity at IC_{20}

3.6 Combined effect CHX and ZnONPs on Mortality at IC_{50}

At IC_{50}, the effects on mortality were shown to be exaggerated. Of the 1:3 ratio, the highest death rate was recorded with an average of 53.6 ± 0.9% and was even more than the equimolar one to one 1:1 ratio, and any of the ZnONPs-rich groups (Figure. 6). The Combination index for the 1:3 ratio was 1.70, thus establishing antagonism to a lesser extent than IC_{20}. The implication here was that while the ZnONPs compromised CHX toxicity by the extended manner, the high relative amount of CHX was still able to dominate all biological consequences at this concentration.

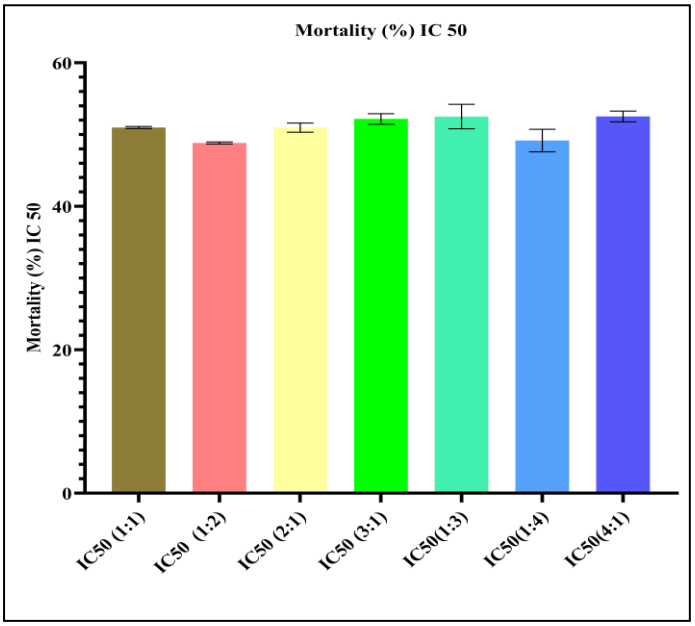

Figure 6. Mortality (%) in zebrafish embryos combination of Chlorhexidine (CHX) and Zinc Oxide Nanoparticles (ZnONPs) at IC_{50}, expressed as mean ± SD from three

3.7 Combined effect CHX and ZnONPs on Teratogenicity at IC_{50}

At IC_{50}, the teratogenic effects became pronounced, especially in the 1:2 and 1:3 combinations. Teratogenicity rates went above 40%, with the most prominent observations being swelling of the body, failure of appendage differentiation, and incomplete elongation of the body axes (Figure. 7). ZnONPs-rich ratios (3:1, 4:1) still retained low frequencies of abnormalities, which may indicate interference with CHX uptake or attenuation of ROS-induced developmental toxicity. Once more, CI values above 1.5 for all ratios indicated that the interactions were antagonistic; however, the biological consequences arising from CHX-rich combinations reached severe levels.

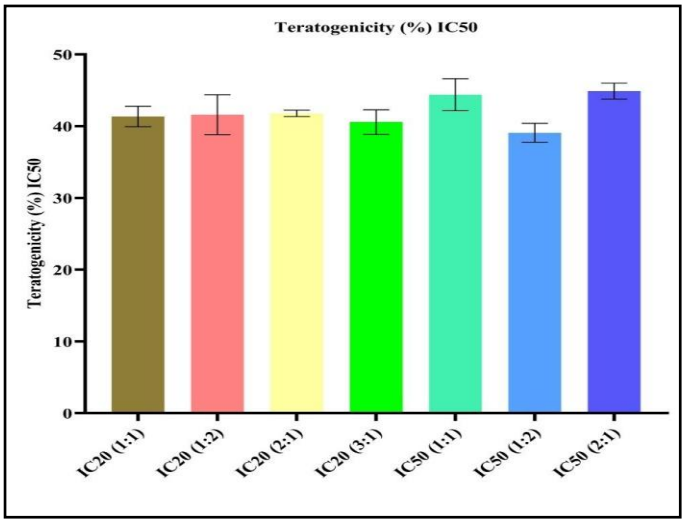

Figure 7. Teratogenicity (%) in zebrafish embryos combination of Chlorhexidine (CHX) and Zinc Oxide Nanoparticles (ZnONPs) at IC_{50}, expressed as mean ± SD from three independent experiment

3.8 Combined effect CHX and ZnONPs on Heart Rate Reduction at IC_{50}

Suppression of the heart rate was highest at 50%. The 1:3 ratio produced an average heart rate decrease of over 33%, emphasizing the ability of CHX to disrupt neuromuscular and mitochondrial functions (Figure.8). 1:1 and 1:2 ratios showed a considerable reduction (about 31%) while ratios dominated by ZnONPs indicated a lack of prominent effects. Such behaviours suggest that CHX-richer mixtures possessed significant physiological stress even in the antagonistic mixture.

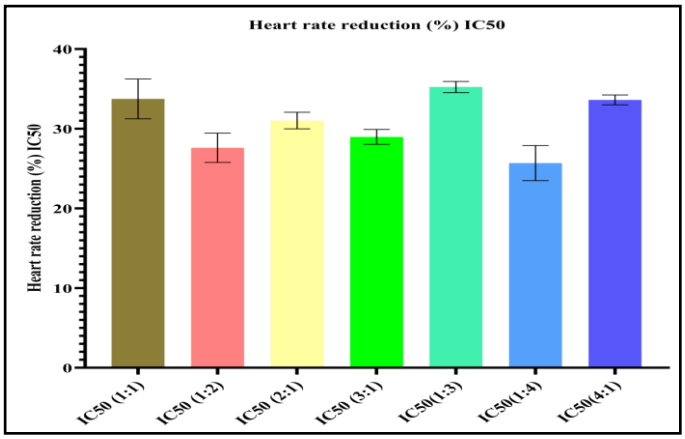

Figure 8. Combined effect CHX and ZnONPs on Heart Rate Reduction at IC_{50}

3.9 Combined effect CHX and ZnONPs on Spinal Cord Deformity at IC_{50}

Spinal deformities increased at IC_{50}, where 1:3 and 1:4 combinations induced the highest deformity rates. Notable deformities included severe dorsal bending, torsion on the longitudinal axis, and poorly defined outline of the carapace (Figure.9). The higher severity and frequency of skeletal deformities produced by CHX-rich mixtures imply that protein conformation and differentiation of embryonic tissues could be particularly susceptible to CHX toxicity, with some degree of protection offered by ZnONPs at this intermediate concentration.

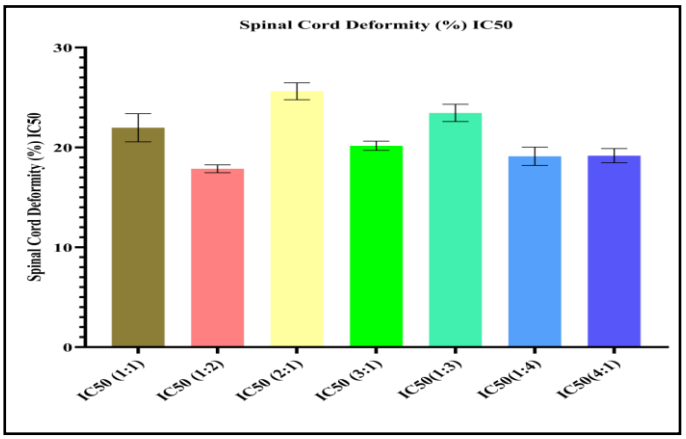

Figure 9. Combined effect CHX and ZnONPs on Spinal Cord Deformity at IC_{50}

3.10 Combined effect CHX and ZnONPs on Mortality at IC_{70}

At IC_{70}, most ratios experienced a plateau in mortality, which was at 51.1 ± 0.5%. The 1:2 combinations produced the highest mortality within this group Figure. 10). The CI for this ratio equated to 1.60, hence further supporting the antagonistic course, while the biological effect remained significant. This implied that interactive buffering is not entirely worthless at elevated chemical interaction; however, at very high concentrations of CHX, it was indeed the concentration that can override chemical interactive buffering apart.

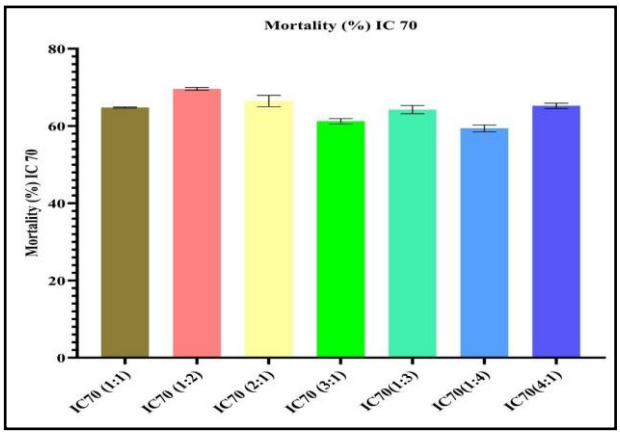

Figure. 10 Mortality (%) in zebrafish embryos combination of Chlorhexidine (CHX) and ZnONPs

3.11 Combined noparticles (ZnONPs) at IC_{70}, expressed as mean ± SD from three independent experiments effect CHX and ZnONPs on Teratogenicity at IC_{70}

The teratogenic effects were very strong at IC_{70}, especially in the 1:2 and 1:3 ratios, and these deformities included organ protrusions and shortened thoraxes that were similar to those evident at IC_{50} (Figure. 11). Combinations with the possible predominance of ZnONPs, for instance, 3:1 and 4:1 ones decreased the percent occurrence of deformities and suggested that ZnONPs might also be able to partially interfere with the CHX teratogenic pathways through reactive oxygen species (ROS) scavenging or membrane stabilization.

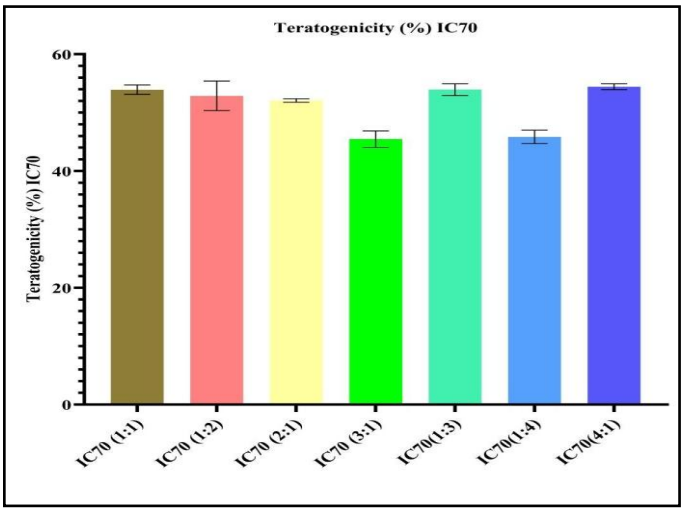

Figure 11. Teratogenicity (%) in zebrafish embryos combination of Chlorhexidine (CHX) and Zinc Oxide Nanoparticles (ZnONPs) at IC_{70}, expressed as mean ± SD from three independent experiments

3.12 Combined effect CHX and ZnONPs on Heart Rate Reduction at IC_{70}

The noticeable one reached its peak at IC_{70} then it never lowered down by over 34% by CHX-rich combinations (Figure. 12). The most reliable suppression was shown by the 1:2 ratios proving that physiological stress heightens with more dose exposure and not linearly reduced by adding more ZnONPs.

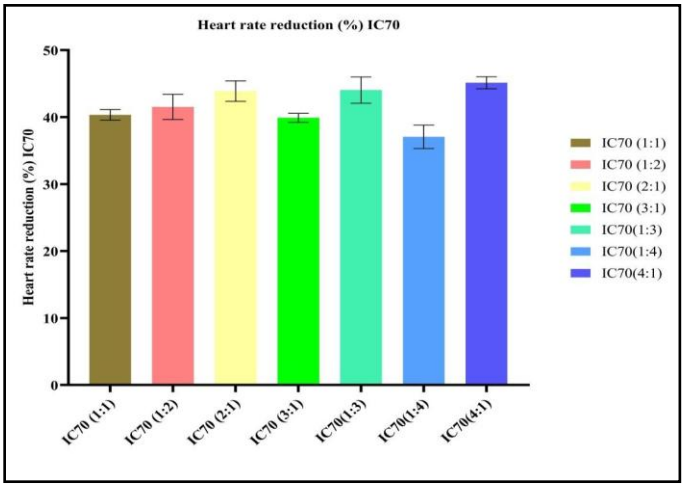

Figure 12. Combined effect CHX and ZnONPs on Heart Rate Reduction at IC_{70}

3.13 Combined effect CHX and ZnONPs on Spinal Cord deformity at IC_{70}

Spinal deformity rates at IC_{70} remained ca. 20.5 ± 1.5%, showing a slight reduction from IC_{50} in certain combinations (Figure. 13). The reason for this could also be explained by the removal of most heavily deformed individuals by lethality or delayed onset of structural abnormalities at very high concentrations. Still, the continued observation of skeletal disruptions at this concentration indicates ongoing developmental interferences.

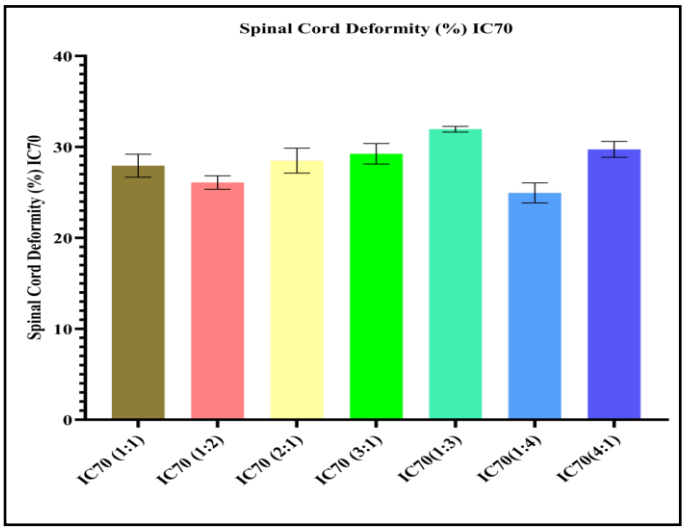

Figure 13. Combined effect CHX and ZnONPs on Spinal Cord Deformity at IC_{70}

3.14 Combination Index

The interactions observed in mixtures of ZnONPs and chlorhexidine at various ratios seemed to depend on concentration in a concentration-dependent manner. At IC_{20} and IC_{50}, most ratios presented CI values greater than one, indicating antagonistic interactions especially the 1:1 and 1:2 combinations (Figure. 14). At IC_{70}, however, the CIs for the 3:1 mixture increased sharply to almost 9, indicating strong antagonism. On the other hand, combinations favoring CHX, such as 1:3, 1:4, and 4:1, showed a significant decrease in CI at IC_{70} with values falling below one, indicating the onset of synergistic interactions at higher concentrations. Thus, an understanding is gained that while at lower concentrations antagonism prevails, at the elevated toxic stress levels synergism will occur in CHX-heavy combinations.

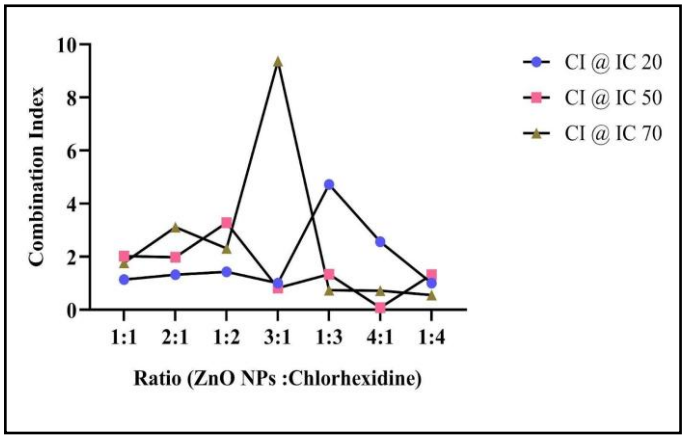

Figure 14. Graphical representation of the combination index (CI) across different CHX and ZnONPs ratios, illustrating synergistic, additive, or antagonistic interactions based on CI values

3.15 Synergism

In the determination of synergism, CHX and ZnONPs were evaluated separately and in combination at various concentrations ranging from 0.001, 0.01, 0.05, and 0.5 ppm, respectively. At the 0.001 ppm concentration, CHX and ZnONPs demonstrated effects of 5.96% and 6.62% individually, amounting to an expected additive effect of 12.58%; the actual observed effect in their combination was, however, 13.98%, thereby indicating enhancement in synergy (Figure. 15). This pattern persisted for all tested doses, with the most significant synergy noted at 10 µM, where the expected additive effect was 73.90% and the effect of combination skyrocketing to 121.87%, giving a net synergistic boost of 47.97%. On the average, the synergistic combination recorded mean augmentation by 14.61% over the expected additive effect, thereby substantiating its capability to enhance efficacy over time.

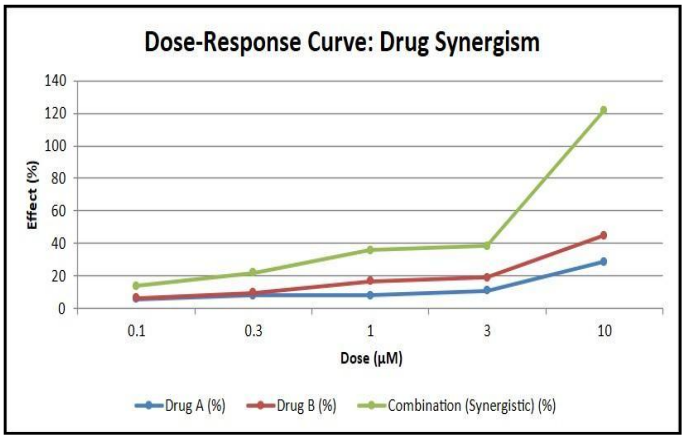

Figure 15. Graphical representation of drug synergism, showing the interaction profiles of CHX and ZnONPs across tested ratios, with classification into synergistic, additive, or antagonistic effects based on combination index analysis

3.16 Antagonism

Contrarily, the combined attempts of two antagonizing drugs showed a different presence. At the lowest concentration of 0.1 µM, Drug A and Drug B induced effects of 4.10% and 3.42% respectively, with the expected additive effect being 7.52%; the drug combination was only 3.83%, indicating an antagonism blocking the efficacy (Figure. 16).. Such antagonistic interaction remained consistent and only strengthened with increase in the dosage; at 10 µM, the individual effects summed to a total of 183.57% and the combined effect was a mere 68.77%, exerting an antagonistic suppression of -114.80%. The mean antagonistic suppression force across all concentrations was calculated to be -53.29%, proving beyond any reasonable doubt that there existed an antagonistic relationship between the two compounds.

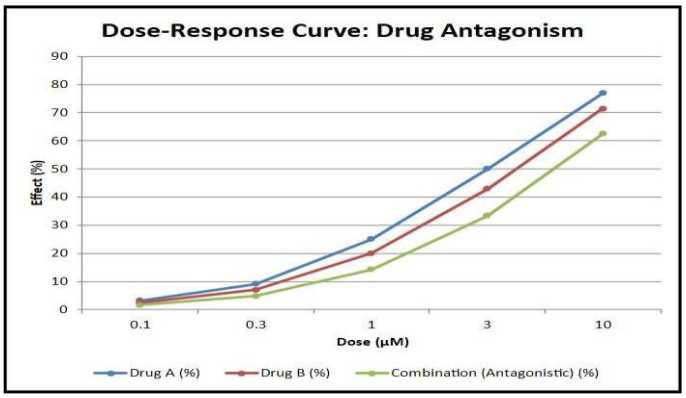

Figure 16. Graphical representation of drug antagonism, depicting combinations of CHX and ZnONPs that exhibit reduced efficacy compared to expected additive effects, as determined by combination index analysis across tested ratios.

These results demonstrated the profound real-life implications of drug interactions and emphasize the vital need for preclinical combination assessments to derive full predictions about clinical effectiveness and safety. One-way ANOVA followed by Tukey's post hoc testing ($p < 0.05$) was used to rigorously test the differences between measured and predicted Bliss values. Most especially, intermediate concentrations (0.01 – 0.05 ppm) showed considerable differences where antagonistic effects were maximal in all experimental combinations. This justified the function of the Bliss model as a robust forecasting mechanism for expectation of mixture toxicity in aquatic toxicology.

This study showed that mixtures between ZnONPs and CHX brought about toxic effects in *zebrafish* related to concentration and ratio. Ratios that favored CHX, for example, 1:2 and 1:3, consistently reported the highest mortality rates, developmental malformations, and physiological derangements at IC_{20}, IC_{50}, and IC_{70}. Although these CI values allotted all those interactions as antagonistic, the actual biological effects remained significant, especially in CHX-rich mixtures. The resultant minimal buffering effects of ZnONPs above high doses therefore serve to prove the necessity

of all these interaction models and endpoint-specific outcomes in getting precise ecological risk assessments for mixed chemical exposures.

DISCUSSION

Recent studies, however, have increasingly classified ZnONPs into the much-less toxic categories among the typical heavy-metal-based nanomaterials. But significant mortality and teratogenicity in aquatic species including zebrafish occur through increased concentrations, indicating the environmental dilemma if they exist in accumulating concentrations in water bodies[18-21]. Previous study suggested that ZnONPs might induce toxicity via many different mechanisms, including oxidative stress mechanisms; induction of reactive oxygen species (ROS); free zinc ion generation due to dissolution of the nanoparticles; destruction of cellular membranes; and possible external uptake or internalization of the nanoparticles[3,22]. The LC_{50} values for ZnONPs indicated in literature for zebrafish generally vary within a range of 6-12 mg/L (96h). However, any variations regarding formulations or conditions of exposure (i.e., smaller particle size or more sensitive exposure times) can yield lower LC_{50}s, sometimes approaching the low µg/L ranges as suggested by your study[18].

Though chlorhexidine (CHX) has been observed to cause acute and sublethal toxicity to a number of aquatic species with EC_{50}/LC_{50}s from some micrograms per liter to low milligrams per liter depending on the test organism and endpoint) research has equally placed it as among the emerging contaminants in wastewater and the receiving water bodies, thus confirming its bioaccumulative nature and persistence[23,24].

Mixture toxicity is one great avenue through which to advance science, and the present study addressed the matter of mixture toxicity by co-exposing zebrafish to ZnONPs and CHX, assessing immobilization across binary ratios, and implementing the Bliss Independence model. In previous studies using Bliss independence and Loewe additivity approaches, it was consistently found that exposure of engineered nanomaterials with organic pollutants, such as biocides and pharmaceuticals, produces synergistic or antagonistic toxic behaviors rather than mere additivity[25,26]. Usually, synergism poses greater ecological risks because it can significantly amplify effects more than expected from exposure to single agents, thus negating the sufficiency of traditional additive models in regulatory frameworks[3,18]. Both field and laboratory research, increasingly, have been demonstrating that these interactions are often concentration dependent and mechanistically related to modification in bioavailability, internalization, or transformation of one chemical when in the presence of the other. Continuous or repeated mixture exposures are likely to cause more intense or longer-lived effects at the population or community level, thus accentuating the need for chronic and multi-endpoint approaches[5].

Unlike previous studies-almost exclusively focusing on single agents or limited endpoints-current approach was consistent with thrusts pushing for mixture assessments, simultaneous assessment of different environmentally relevant contaminants, and mechanisms like Bliss Independence and response surface modeling in assessing risks. It is reminiscent of regulatory momentum toward the addition of multi-ratio multi-endpoint chronic/multigeneration designs into future risk assessment protocols[27].

This work is restricted to short-term exposure under laboratory conditions. Future research should study the effect of long-term exposure, reproductive toxicity, transgenerational toxicology, and nanoparticle alteration in the real environmental conditions to enhance the risk assessment process.

CONCLUSION

Although combinations of ZnONPs with CHX exhibited antagonistic behavior according to Bliss Independence model, CHX-contaminated mixtures showed biological activity across all endpoints. These findings reveal a limitation of traditional additive models in predicting the ecological effects of complex pollutant mixtures. This study indicates a need for mechanistic toxicology in ecotoxicological assessments, which helps to understand better the ways by which different contaminants interact at the molecular and physiological levels. With the high frequency of engineered nanomaterials and pharmaceutical introductions into aquatic environments, these combination exposure scenarios become ecologically realistic and relevant. Therefore, future risk assessments should incorporate a multi-endpoint, multi-ratio test approach and research on long-term exposure to improve the accuracy of chronic and transgenerational impacts. The use of zebrafish as an indicator species is ideal, given its sensitivities and its function in the ecosystem. This reinforces continued use in environmental monitoring schemes for complex mixtures of pollutants.

DECLARATION

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CONFLICT OF INTEREST

None declared.

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