Fabrication, characterization and toxicity analysis of erbium doped hydroxyapatite

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Abstract:
Introduction
The use of bioactive inorganic materials in many clinical applications has garnered considerable attention. The demand for synthetic biomaterials to restore or/and replace bone tissue lost to injury or disease, which has significantly expanded in recent years, serves as an excellent illustration of the necessity for such materials.Erbium-doped hydroxyapatite (Er-HAp) is a promising material with potential applications in biomedical and optical fields due to its unique properties. It has been a new source to develop tissue engineering using erbium in addition to depict bioluminescence and biomineralization properties.
Materials and methods:
Erbium doped hydroxyapatite was synthesized and subjected to EDX, XRD, ATR-IR and SEM analysis for analyzing the structural and mechanical properties of the synthesized novel complex. The toxicity analysis of the complex was done with zebra fish.
Results:
The formed complex confirmed an in-depth analysis of the crystals formed and were further studied using ATR-IR and XRD analysis. In the zebrafish toxicity analysis, hydroxyapatite doped with erbium did not exhibit any toxicological effects.
Conclusion:
Erbium doped hydroxyapatite can be used as an efficient bone grafting biomaterial for bone regeneration. Future studies would be done invitro and invivo to assess its efficacy in bioactivity when used as a biomaterial for guided bone regeneration.
Keywords- Erbium, Hydroxyapatite,bone regeneration, Toxicity

Introduction:

In the pursuit of enhancing skeletal repair and reinstating skeletal function, bone regeneration encompasses a meticulously orchestrated series of biological processes referred to as bone induction and conduction. These processes entail various cell types and intricate intracellular and extracellular molecular signaling pathways, unfolding in a well-defined temporal and spatial sequence. While materials like autografts, allografts, and alloplasts/synthetic biomaterials have traditionally been utilized for bone regeneration, the surge in demand for synthetic biomaterials to address bone tissue loss due to injury or disease underscores the imperative need for such materials (Culp, 1998). Hydroxyapatite is widely employed as a bioceramic in numerous therapeutic applications, including bioceramic coating, bone tissue engineering, drug and gene delivery, and dentistry applications, because of its superior osteoconductive properties(Chen et al., 2021; Neacsu, Stoica, Vasile, & Andronescu, 2019; Zhang, Jo, Chen, Hontsu, & Hashimoto, 2022).The addition of erbium ions to the hydroxyapatite structure has been found to enhance its mechanical and biological properties (Minh, 2022). The current study is an analysis done to enhance the osteoconductive property of hydroxyapatite with addition of erbium ions for bone regeneration.

The erbium elements on the other hand are currently receiving a lot of attention because they can be used to create photoluminescent materials for biotechnological and/or electrical research(Neacsu et al., 2019; Zhang et al., 2022). They have also been used as a potential replacement for organic fluorophores and quantum dots in the biomedical field and are currently being researched as a new class of luminescent optical labels(Ceballos-Jiménez et al., 2018). Luminescent materials, which primarily consist of fluorescent molecules (organic mainly) and semiconductor nanoparticles, have been thoroughly investigated for applications in biological staining and prognostics in the biomedical area(Neacsu et al., 2019).Additionally, by substituting various luminescent rare-earth elements for the calcium (Ca2+) ions in hydroxyapatite, luminescent characteristics could be added(Kumar, Rehman, & Chaturvedy, 2017).Erbium- 169 also has a potency to emit beta particles which kill the metastasized cancer cells and tissues. This study marks the initial analysis of how erbium ions, a rare earth element, could have an impact on bone regeneration. There are no scientific evidence of use of erbium ions with hydroxyapatite in application to bone regeneration (Chen et al., 2021; Neacsu et al., 2019; Zhang et al., 2022).Hence this research is an innovative approach of incorporating erbium ions onto the hydroxyapatite matrix and to analyze its physicochemical characteristics and toxicity.

Materials & methodology:

The study was conducted at the blue purple and green lab of the Department of Periodontics, Saveetha Dental College and Hospitals. Before conducting the study, the institutional ethical review board at Saveetha Dental College and Hospitals, Chennai, India examined and approved the clinical protocol for study.

Study procedure:

1. **Preparation of Erbium doped HAp**

   2.1 g of cetyltrimethylammonium bromide, 0.099 mol of calcium nitrate and 0.01 g of erbium were dissolved together in a beaker to which 67 g of diammonium hydrogen phosphate was added and the resultant precipitate was washed using acetone or ethanol. The precipitate was then kept at 400 degree celsius for 3 hours. The final obtained powder was then stored in an eppendorf tube. The presence of the formation of the desired product and its morphology was tested using EDX, XRD, ATR-IR and SEM analysis respectively.

2. **SEM, EDX, XRD and ATR-IR analysis:**

SEM analysis was carried out to determine the morphology of the formed final product, and EDX analysis was used to provide elemental identification and quantitative compositional information. XRD analysis was performed as it provides useful information on the crystal phase, lattice constant, and average particle size of nanoparticles synthesized. And finally ATR-IR to identify whether the prepared mixture is in a solid or a liquid state which does not need to be prepared for further analysis.

In Vitro toxicity analysis:

Using the obtained erbium doped hydroxyapatite, an in vitro toxicity study was conducted against zebrafish embryos. Using 25 marked zebrafish eggs at various ratios (25, 50, 75, 100, and 200 µg/ml) of the synthesized erbium doped hydroxyapatite in solution, the toxicity was evaluated based on the OECD-203 protocol.After that, the eggs were placed in separate wells to continue developing into their heads, tails, and eyes, which were all clearly visible every 24 hours under a 40 × microscope. In order to prevent contamination in the solution during the assay, the water was kept at room temperature at all times, and the live and dead fish were checked every 24 hours. The rates of embryonic hatching and mortality were determined every 24 hours.

RESULTS:

The synthesized erbium doped HAp particles are shown in **Figure 1.** Total of three different analyses were done to characterize the presence of erbium doped HAp particles; those include EDX, SEM, XRD and ATR-IR. **Figure 2** depicts the outcome of EDX analysis, in which the presence of the basic trace elements of the main synthesized material resides. The analysis confirms the formation of Erbium mineral along with other trace elements like calcium and phosphorus. The spectrum 16 consists of 37.5% of carbon, 31.5% of oxide, 30.9% of calcium, 0.1% phosphorus and 0.0% erbium in weight. **Figure 3** shows the clear morphology of the formed particle, the particle synthesized has a crystalline structure which has dense and coarse particle size, having the morphology of stacked sheets and can be implied in different advanced scientific imaging which is commonly used for high resolution material activity identification. **Figure 4** depicts the outcome of the XRD analysis, the incidence of high peaks describes the crystalline structure of the synthesized particle. XRD analysis in which the crystallographic structure of the material is identified, and the importance of the micro and nano structure in material science. The crystallographic structure of the synthesized material has a different diffraction pattern because of the material's particular chemistry and atomic organization. **Figure 5** and its values in **Table 1** shows the ATR-IR analysis of the material, where the synthesized particle has been formed without any further preparation required to test the characterization of the material. At the wavenumber of 1135 the lewis ion that is HPO42-, at wavenumber 1071 carbonate was present and at wavenumbers 1029, 921, 719 phosphate ion was present. At different wavelengths different elements were traced and found, the basic elements include carbonate and phosphate.

The in vitro toxicity tests were carried out by diluting the erbium doped hydroxyapatite ratios with Hank's solution. After being exposed to hydroxyapatite doped with erbium, the embryonic mortality of viable zebrafish eggs was investigated. Using a light field microscope with 40 × resolution, mature embryos at 24 and 48 hours post fertilization as well as eggs exposed to erbium-doped hydroxyapatite were observed. The derived result verified that during the initial treatment phase, a greater number of eggs exposed to hydroxyapatite doped with erbium exhibited a delayed hatching and less mortality.

A microscope was used to clearly identify the mature embryos treated with erbium doped hydroxyapatite after 72 hours, allowing for the identification of malformations and complete genesis of the tail, head, and eyes. In contrast, samples exposed for 92 and 120 hours demonstrate that most eggs have hatched as shown in the **figure 6**. The toxicological investigations on formation-stage embryos using hydroxyapatite doped with erbium reveal a markedly reduced mortality rate. The death of zebrafish larvae is just 10%, which confirms that the erbium doped hydroxyapatite is of negligibly less in toxicity as shown in **Figure 7**. Hence in the zebrafish study, hydroxyapatite doped with erbium did not exhibit any toxicological effects.

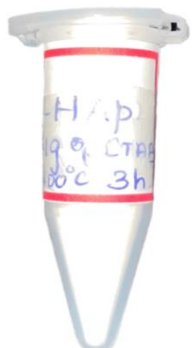

Figure 1: Erbium doped HAp material synthesized

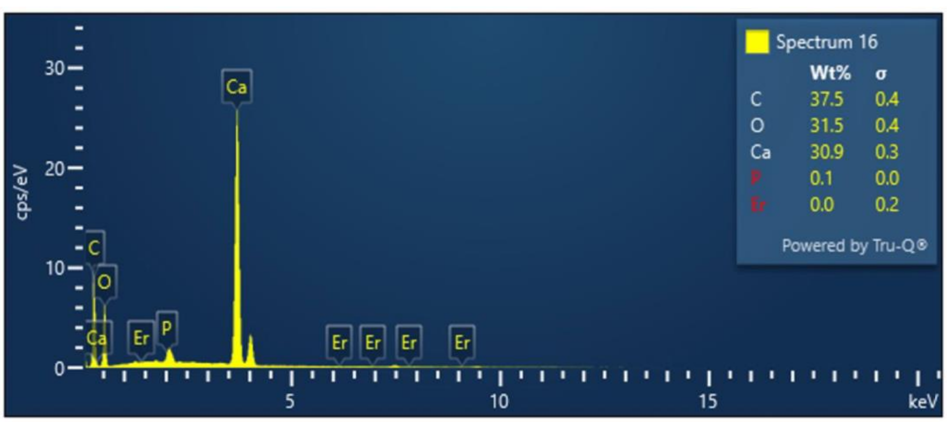

Figure 2: EDX analysis confirming the presence of various elements with trace elements.

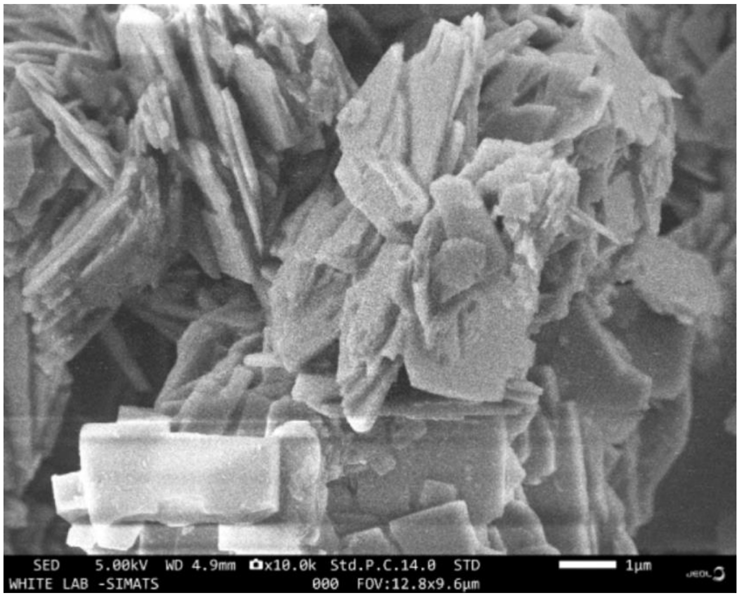

Figure 3: SEM analysis showing the morphology of the material synthesized, being coarse particle with crystalline structure and has the shape of stacked sheets.

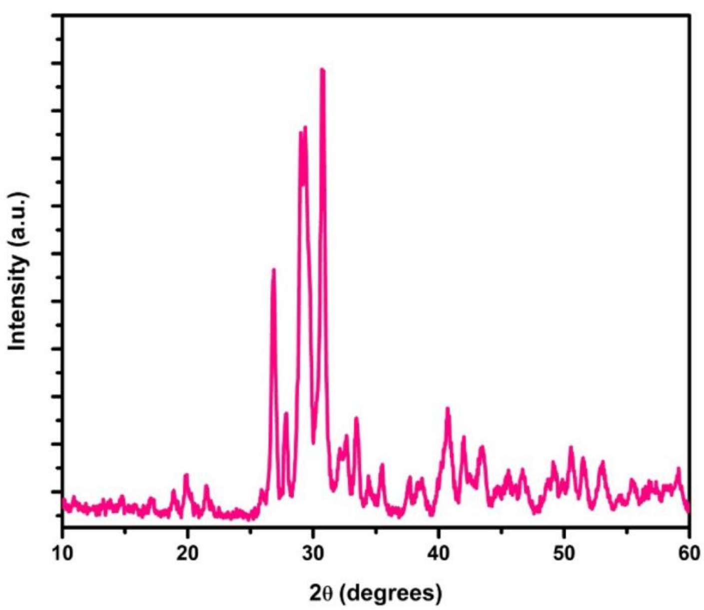

Figure 4: XRD analysis showing the formation of erbium doped hydroxyapatite with crystalline structure denoted by high peaks.

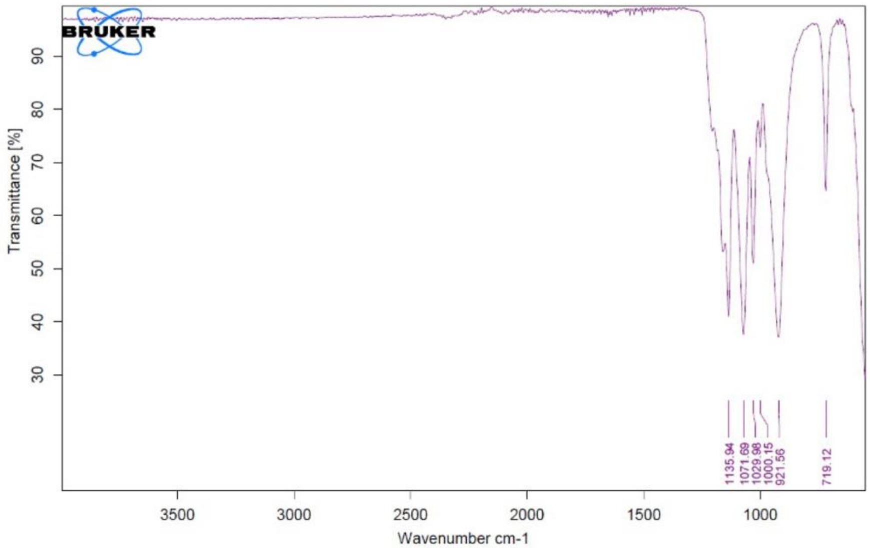

Figure 5: ATR-IR analysis showing the formation of various elements at different wavelengths. Table 1 depicts the elements found at its corresponding wavelengths.

Wave number (cm^{-1})	Interpretation	References
1135	HPO_4^{2-}	[1]
1071	Carbonate	[2]
1029	Phosphate	[3]
921	Phosphate	
719	Phosphate	

Table 1: depicts the elements found at its corresponding wavelengths in the synthesized material.

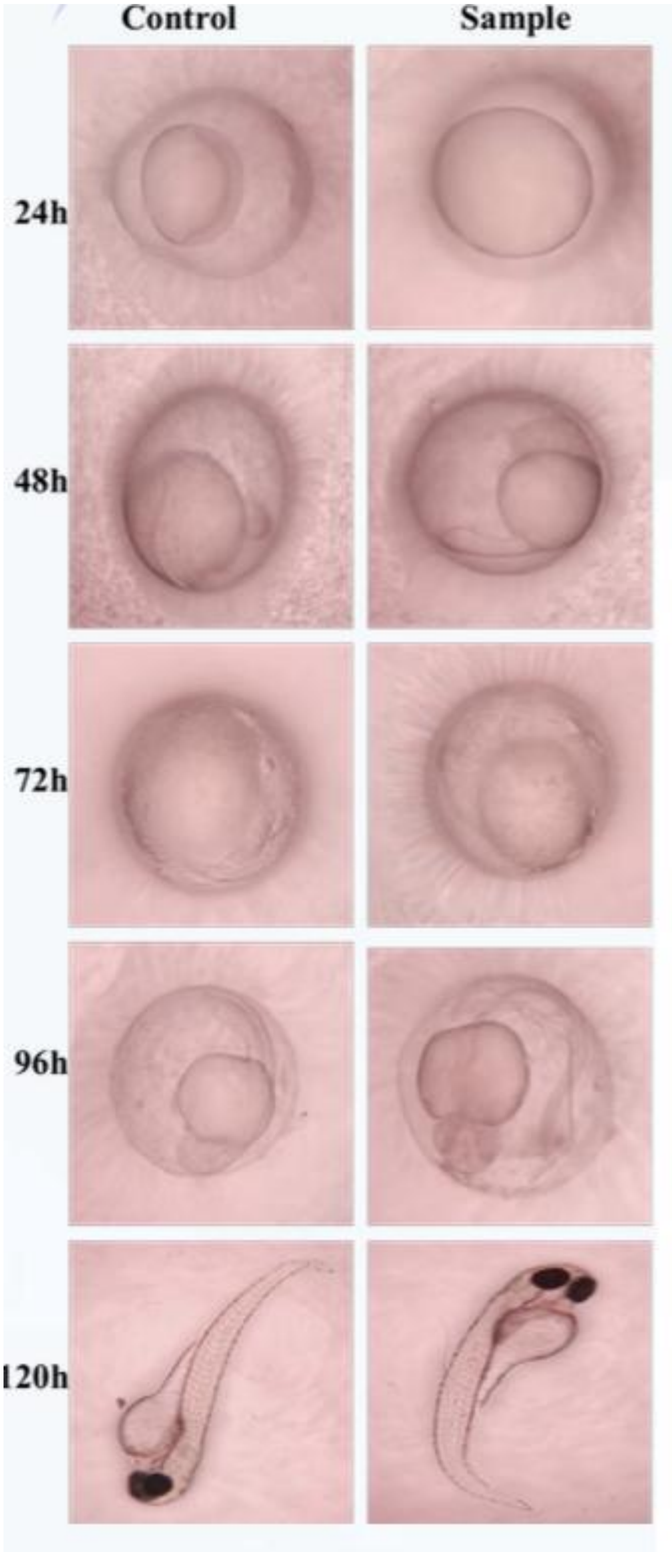

Figure 6: The toxicity analysis of erbium doped hydroxyapatite with zebrafish larvae for about 120hr.

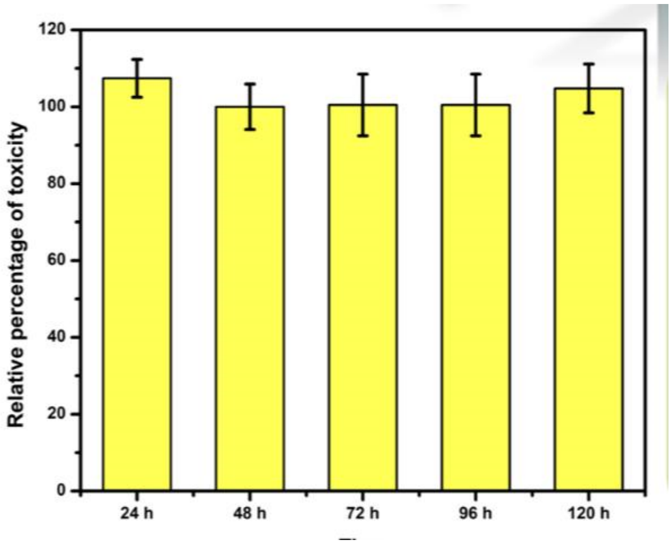

Figure 7: Graph illustrates that the death of zebrafish larvae is just 10%, which confirms that the erbium doped hydroxyapatite is of negligibly less in toxicity.

Discussion:

Characterization of Erbium-doped hydroxyapatite involves several analytical techniques to assess its structural, morphological, and optical properties. X-ray diffraction (XRD) is used to determine the crystal structure and phase composition of the synthesized material (Ivanova & Crawford, 2015; Patidar, Patel, & Valand, 2015), confirming the formation of hydroxyapatite and the presence of erbium ions within the lattice. Scanning electron microscopy (SEM) provides information about the morphology and particle size of the Er-HAp crystals (Khan et al., 2014).

The synthesized material is subjected to an electron beam during the EDX analysis procedure, which causes the sample's atoms to release distinctive X-rays. The elemental makeup of the sample is then ascertained by detecting and analyzing these X-rays (Čeh & Gec, 2005; Rehan, Rehan, Sultana, & Khan, 2023). This research aids in understanding the distribution and concentration of erbium within hydroxyapatite particles doped with erbium, which in Figure 2 is clearly found that the erbium particles are present in high concentration in the synthesized material. The synthesized erbium doped hydroxyapatite has been studied for its potential use in biomedical applications such as drug delivery and bone tissue engineering. The addition of erbium ions to the hydroxyapatite structure has been found to enhance its mechanical and biological properties (Minh, 2022). Simultaneous analysis of the material synthesized includes SEM, scanning electron microscopy, is another commonly used method for studying the microstructure of the material. It allows for high-resolution imaging of the particles and can provide information on their size, morphology, and distribution (Minh, 2022; Riaz et al., 2018)17,18(Minh, 2022; Mucalo, 2015; Riaz et al., 2018). SEM pictures showing coarse and crystalline structures made of the spike-like particles were visible in the SEM pictures for the sample(Jayapal et al., 2021) (Suresh & Kaarthikeyan, 2024) . The materials EDX spectra showed no signs of additional elemental contaminants. It was discovered that the oxide content was barely present. The EDX data indicated that an increase in calcium concentration led to erbium deficiency (S et al., 2024) (Soni, Panneer Selvam, Shanmugam, Ramadoss, & Sundar, 2024). Erbium with traces of calcium and erbium doped hydroxyapatite particles have shown promising results in the field of dentistry.

Based on previous Research done in vitro using teleost cells (Ictalarus punctatus). Cell viability was assessed following a 24-hour exposure at different doses, and the PI staining assay yielded results. The DNA content determined by PI staining is directly correlated with the number of dead cells in the culture and contrasted with negative controls because the dye is non-permeate and can only penetrate the membranes of dying or dead cells(Thirumalai, 2018). Additionally, cellular metabolic activity (MTS assay) was measured; the outcomes were expressed as specific metabolic activity and normalized against cell viability. Specific toxicological endpoints were monitored for a duration of 120 hours post fertilization in order to assess the toxicity of nHA to zebrafish embryos(Tithito et al., 2023). Not withstanding the likelihood that fish would not come into contact with this concentration in the wild, a range of 3 to 300 μ g mL − 1 was employed to enable comparisons between in vitro and in vivo findings. The embryonic mortalities caused by exposure to nHA were observed at 24 and 120 hpf, and it was discovered that nHA exposure(Gao, Yang, Lv, Song, & Song, 2018).

Additionally, the hatching rate was noted, and at 72 hpf, a hatching delay was noted. It's interesting to note that both shape and concentration affected the hatching delay. Within 48 to 72 hours post fertilization, control zebrafish embryos hatched from their chorion when cultured at a normalized 28.5 °C freshwater condition. Nevertheless, at 72 hpf, zebrafish embryos exposed to nHA exhibited a significant delay in hatching when compared to the control groups. By 80 hpf, the hatching rate returned to normal, and all of the embryos hatched out at 96 hpf. For instance, only 30–40% of the embryos exposed to nHA-RD at 3 μ g mL − 1 hatched at 72 hpf, compared to nearly 80% of control embryos. Later on (80 hpf and above)(Gao et al., 2018; Pujari-Palmer, Lu, & Karlsson Ott, 2017)(Garapati, Malaiappan, Rajeshkumar, & Murthykumar, 2022).

At 96 hpf, the hatching rate of the embryos exposed to nHA finally reached 100%. It has been previously documented that exposure to ZnO nanoparticles and single wall carbon nanotubes causes zebrafish embryos to hatch later than expected. In contrast to previous reports, our study revealed that the lowest concentration resulted in the highest hatching inhibition, with nHA-ND exhibiting the lowest hatch at 72 hpf (less than 20%) (Figure 4). For nHA-ND, no significant hatching delay was seen at the other higher concentrations between 10–300 μ g mL − 1. In contrast, all testing concentrations (3–300 μ g mL − 1) for nHA-RD demonstrated hatching inhibition, while the lowest hatching rate (approximately 30%) was still observed at the lowest particle concentrations (3 and 10 μ g mL − 1)(de Souza et al., 2023).

The nanoparticles might not be able to cross the chorion at higher concentrations. Studies on the interactions between proteins and nanoparticles have demonstrated that proteins can be highly adsorbed by nanoparticles. The zebrafish hatching enzyme, a metalloprotease secreted from the hatching gland and essential to the breakdown and weakening of the chorion at hatch, may interact with nanoparticles. The hatching enzyme may undergo conformational changes as a result of nHA adsorption, which would impair the enzymes' ability to function. Inhibiting the activity of proteases, nanoparticles have been demonstrated to postpone zebrafish hatching in vivo. possessing a large specific surface area (de Souza et al., 2023; Karunakaran et al., 2023).

Moreover, the developmental delay was noted in embryos cultured with 3 μ g mL − 1 nHA-RD. When compared to the control group, nHA-ND and nHA-RD at the maximum concentration of 300 μ g mL − 1 did not significantly inhibit larval development. At 120 hpf, all samples had fully developed larvae with normal morphology. At 120 hpf, 75% of the embryos incubated with 300 μ g mL − 1 nHA-ND exhibited a bent spine (Figure 5). Axial curvature has been demonstrated to result from exposure to a variety of metals and nanoparticles, such as heavy metals, cadmium selenide nanoparticles, silver nanoparticles, and platinum and silver nanoparticles. Axial deformation has been linked to cardiac function impairment, interference with somite formation, and oxidative stress, among other theories (Tomić, Nikodinović-Runić, Vukomanović, Babić, & Vuković, 2021).

CONCLUSION:

Erbium doped hydroxyapatite nanoparticle was synthesized and verified using EDX analysis, the microstructure of the formed material has coarse particles with crystalline structure. XRD and ATR-IR analysis to ensure the crystalline structure of the formed material and identified the presence of basic elements formed at different wavelengths respectively.Within the limitations of the study the obtained erbium doped hydroxyapatite did not cause any mortality in zebrafish confirming their safety. The fabrication of this novel nano particle can be further investigated for osteogenic potential and used for various biomedical applications.

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Conflict of Interest -Nil

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