**Influence of Prednisolone and Alendronate on the *de novo* Mineralization of Zebrafish**

**Caudal Fin**

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**Medicine Effects on Zebrafish Bony-Rays**

# **Introduction**

Osteoporosis is a chronic degenerative disease of bones, characterized by the imbalance between bone resorption by osteoclasts and the ossification process by osteoblasts during the remodelling process.(1) The increased bone remodelling activity in favour of osteoclasts often leads to reduced bone-mineral density (BMD), high porosity and, consequently, in the deterioration of mechanical properties and high fracture risk.(1–3) As the people’s life expectancy increases,(4) osteoporosis may cause a significant impact on the life quality of elderly people and burden the world’s economy in a short-time.(2,5)

Prednisolone (PN) is a glucocorticoid often used as a treatment to inflammation and its prolonged administration is well known to cause secondary osteoporosis in humans.(6) In order to counteract the adverse effects of the continuous use of glucocorticoids, antiresorptive agents (e.g. bisphosphonates) has been suggested.(7–9) Among the available bisphosphonates, Alendronate (ALN) is a compound that could regulate bone remodelling by promoting osteoclasts apoptosis and osteoblast differentiation.(7,10) The long-term administration of ALN has been shown to increase continuously BMD of the lumbar spine, with no serious adverse effects related to fractures, reversing the osteoporotic phenotype in post-menopausal women.(11) Despite the positive outcomes, little is known of how mineralized tissues, specifically for human diseased bones, are influenced by these anti- and pro-mineralogenic medicines at the multi scale-level. The evaluation of the compositional, morphological and mechanical characteristics is particularly limited.(12) The use of animal models has therefore been proposed.(13,14)

Zebrafish are small teleost fish and have emerged as a suitable model to study anti-(15–18) and pro-mineralogenic(7,19) compounds due to their feasibility for *in vivo* imaging, easy follow up potential, and their conserved bone physiology with humans.(15,20–22) One of the most notorious characteristics of zebrafish is their ability to regenerate fins after resection and repair minor injuries in their skull.(20,23–25) Their caudal fin is composed by ~18 rays of intramembranous dermal bones (lepidotrichia) that, after resection, triggers a process to be repaired, attaining similar shape and function as the inborn appendage.(20,24–28) It has been previously demonstrated that dedifferentiated osteoblasts play a key role in the lepidotrichia’s regenerative process.(27) Conversely, the role of osteoclasts in the fin’s regeneration process is still not clear.(23) However, when PN is administered simultaneously to a regenerative process, the recruitment of these cells to the injured site is irregular,(23,27,29) which may impair the regrowth and properties of the newly formed fin. A commonly used approach to assess the changes caused by osteo-active compounds on zebrafish is by using a fluorescent dye (e.g. Alizarin Red-S (AR-S)) to stain their mineralized tissues.(2) The mineral depletion caused by bone diseases (e.g. glucocorticoid-induced osteoporosis), has been demonstrated to decrease the detected fluorescence signal intensity.(15) On the other hand, increased intensities were observed when bisphosphonate compounds (e.g. ALN) were administered.(19)

The mineralogenic effects of PN and ALN on zebrafish’s skeleton are not novelty,(2,15,17,19) however, little is known on their after-effects in the multi-scale level. Thus, the aim of this study is to evaluate, the appearance, the composition and mechanical properties of zebrafish caudal fin bones, before and after resection, affected, or not, by PN or ALN. The application of resection/regeneration processes, enables the longitudinal evaluation of the same individual, which may minimize any potential effects caused by each individual characteristic (e.g. genetic variation) and also complies with the 3 R’s in ethical Guidelines for animals' research.(22,30) Understanding how medicines influence the caudal fin bony rays’ properties in zebrafish could further the understanding on the patho-physiological of diseased mineralized tissues. The null hypothesis to be tested is that the different medicine treatments do not affect the properties of zebrafish caudal fin bones.

# **Materials and methods**

## **Zebrafish maintenance**

Adult three-month-old wild-type AB strain zebrafish (n = 46), with average length of 30.73 ± 4.32 mm, were obtained from the Taiwan Zebrafish Core Facility at Academia Sinica (TZCAS). The fish were maintained inside tanks in recirculating water system under a 14/10 hours light/dark (l/d) cycle at 28 °C and were fed twice a day, as described in the Zebrafish Book,(31) until they were sorted to experimentation. The experimental use of zebrafish was approved by the Experimental Animal Care and Use Committee of NTHU (IACUC approval number: 10048). The steps used to evaluate the zebrafish caudal fins in this work are represented in the Supplemental Figure 1.

## **Lepidotrichia imaging and glucocorticoid-induced osteoporosis model**

Thirty-one fish (n = 31) were randomly selected from the initial forty-six and were immersed for 15 minutes in a 0.01% Alizarin Red-S solution (Sigma Aldrich; Missouri, MO, USA) with its pH adjusted to ~7.4 with KOH solution. The fish were rinsed in fish tank water for three times every 5 minutes to remove the excess of AR-S staining.(21) Then, the fish were anaesthetized individually in a 200 mL solution containing 70 ppm of Tricaine (MS-222;Sigma Aldrich; Missouri, MO, USA), adjusted to pH ~7.2 (± 0.1) with sodium hydroxide (NaOH), 130 µL of isoflurane (Alfa Aesar; Loughborough, Leicestershire, UK) and ethanol (isoflurane:ethanol = 1:9), as described elsewhere.(32) The anaesthetized fish were placed carefully onto a cell and tissue culture disk (Biofil; Kaohsiung, Taiwan) and the tiles images of the bright field (T-PMT-T3) and fluorescent light (Cy3-T3) of the caudal fin bony rays were taken with an inverted confocal microscope (LSM 800; Carl Zeiss, Oberkochen, Germany). The pinhole size was set to 4.49 A.U., with a bit depth of 8-bits and the detection gain were set to 170 V and 680 V (T-PMT-T3 and Cy3-T3, respectively). The pictures obtained were labelled as zero days (0 d). The other fifteen fish were raised separately, in fish tank water only, for later use.

After being imaged, the zebrafish were put individually into 120 mL of fish tank water containing PN (Sigma Aldrich; Missouri, MO, USA) previously dissolved in 0.1% of dimethyl sulfoxide (DMSO; J.T. Baker; Pennsylvania, PA), in a final concentration of 125 µM. During 14 days, 1/3 of water with the medicine was changed daily. After 14 days (14 d), the fish were AR-S stained, anaesthetized and analysed again by an inverted microscope using the same parameters as described previously. The caudal fins images had the pixel intensity of the ventral lobe bony-rays measured with ImageJ 1.52a (Wayne Rasband, NIH, Bethesda, MD). The pixel intensities were then transformed into percentage and both groups of images were compared. Five fish died naturally during the treatment.

**First fin resection**

The fish not previously exposed to any medicines (n = 15) were sorted and put individually into containers with fish tank water only. Individually, both the untreated and fish with osteoporotic phenotype (n = 26) were anaesthetized and the caudal fin resection were performed ~3 segments proximal to the first lepidotrichia bifurcation. The fins amputated from the group of untreated fish were labelled as the control (CTRL); the fins from the group with osteoporosis were labelled as GIOP. The fish were put into a recovery basin containing fish tank water only before the treatment started.

## **Medicine administration and de novo mineralization**

The amputated living fish were put into numbered containers, and were assigned into three groups according to the treatment proposed. The fish with osteoporosis were divided into two treatment groups: Thirteen fish (n = 13) were assigned to tanks filled with 120 mL of fish tank water only, while the other half (n = 13) were added to tanks with 120 mL water containing ALN (Alfa Aesar; Loughborough, Leicestershire, UK) in a concentration of 30 µM. The untreated fish (n = 15) were put to regenerate in tanks containing 120 mL of fish tank water only. The zebrafish remained in numbered containers for 21 days, and 1/3 of the water with and without medicine were refreshed daily. The regeneration process for each fish was followed up, and images were recorded at 1-, 4-, 7-, 11-, 14- and 21-days post amputation (dpa). In each of the timepoints, AR-S staining, water rinsing and anaesthetic procedure were performed individually in each fish; the images were obtained with an inverted confocal microscope, as described previously. Before 1 dpa analysis was performed, seven fish (n = 7) with osteoporotic phenotype died naturally (three from the group assigned to fish tank water only and four fish from the ALN intervention group).

The caudal fin bony rays *de novo* mineralization was quantified in accordance to the parameters suggested by Cardeira *et al.*(20) A summary of these parameters is shown in Table 1A. Using ImageJ, the values extracted from the images analysed were transformed into distance or area (mm or mm2, respectively) by calibrating the pixel values with the scalebar showed in the images, prior the calculations were performed.

## **Second fin resection**

After 21 dpa, the regenerated fins were resected using procedures as described previously. The fins extracted from the fish immersed in fish tank water only (no previous disease condition) were labelled as CTRLREG; the caudal fins amputated from the group of fish that previously developed osteoporotic-like bones, and were let to regenerate in fish tank water only, were called GIOPREGEN and, finally, the fish with osteoporosis condition treated with ALN were labelled as ALNREGEN. A summary of the treatment groups proposed are shown in Table 1B.

## **Mineral morphology and content**

The amputated zebrafish caudal fins (n = 3; each group) were put onto microscope glass slides and were bleached with 5% sodium hypochlorite solution (NaOCl; J.T. Baker; Pennsylvania, PA) in order to remove external tissue and fatty components.(33) The bleaching solution was washed out with deionized water and the fins were dehydrated in ethanol series (50-, 75-, and 100%) for 15 minutes sequentially. The exposed bones were then separated into proximal and distal regions by performing a section ~2 segments proximal to the cleft. The proximal and distal parts were rapidly frozen under liquid nitrogen and crushed into small particles with mortar and pestle. The still frozen particles were critical-point-dried (CPD) in a Samdri-795 critical point dryer (Tousimis®; Maryland, MD) and evaluated. The morphology of proximal and distal regions of the fins were observed using a Scanning Electron Microscope (SEM) SU8010 (Hitachi; Chiyoda, Japan) equipped with an Energy-Dispersive X-ray Spectroscope (EDS). The Calcium (Ca) and Phosphorus (P) atomic percentages, were recorded in five different sites using EDS. The areas of analysis were fixed to 7 × 7 µm2 and the equipment voltage was set to 15 kV for both characterization methods.

## **Raman spectroscopy**

Raman spectra were obtained with a Raman spectrometer iHR550 (Horiba Scientific Ltd.; Kyoto, Japan) equipped with a laser confocal microscope IX71 (Olympus; Tokyo, Japan). A 10x magnification lens and a laser source with wavelength of 632.81 nm were used and the equipment acquisition time was set to 20 s with 3 co-additions. One amputated caudal fin (n = 1), with similar size and with eighteen lepidotrichia each were selected and transferred to opaque 3-D printed polymer substrates. The bones were exposed with NaOCl and subsequently dehydrated in ethanol series. A total of 96 equidistant points (~700 µm distant) had the spectrum recorded and the area under the phosphate peak (~960 cm-1) were calculated using LabSpec 6 software (Horiba Scientific Ltd.; Kyoto, Japan). The integrated values were plot as a heat colour map relatively to the position analysed.

## **Cross-section procedure**

Two amputated fins of each group (n = 2) were gently placed into a cryomold (TissueTek®; Sakura Finetek Europe B.V., Netherlands) and were totally covered with Optimal Cutting Temperature (OCT) compound (TissueTek®; Sakura Finetek Europe B.V., Netherlands). The compounds were indirectly frozen using liquid nitrogen, covered with aluminium foil and stored at -21 °C until used. The blocks were cut into 30 µm thick slices, perpendicularly to the bony rays’ direction growth direction, at -21 °C, with a microtome CM3050 S (Leica Microsystems GmbH; Wetzlar, Germany), as described elsewhere.(34) The used thickness is over the minimum standard accepted to sub-micron analyses.(35) The sliced samples were fixed in properly labelled microscope glass slides and the excess of OCT was washed using phosphate-buffered solution (PBS) with pH of ~7.4, formulated as described by Chazotte.(36) One of the cross-sectioned samples was used in the surface roughness analysis, while the other was used for mechanical evaluation.

## **Surface profile and roughness**

The surface profiles of sliced samples were observed using an ICON® Atomic Force Microscope (AFM; Bruker, Massachusetts, MA). The AFM was operated in tapping mode in air using a non-conductive silicon nitride (Si3N4) tip with a nominal radius of 4 µm (NANOSENSORS™; Neuchâtel, Switzerland). The spring constant was 42 N/m with resonant frequency of 330 kHz. The equipment was set to a scan rate of 0.99 Hz and the images were captured with a resolution of 256 pixels/line. The third lepidotrichia from dorsal part was used in this technique. One slice between the 2nd and 3rd segment proximal to resection plane, and other between the 2nd and 3rd segment near to the distal tips, had the surface profile and roughness recorded. Five areas of 2 µm2 were measured and the average value of Ra was calculated with the software NanoScope Analysis v1.4 (Bruker, Massachusetts, MA).

## **Mechanical evaluation**

Three consecutive slices from each proximal and distal region had their mechanical properties evaluated. A Tribo-Indenter (TI 980; Bruker, Minnesota, MN) equipped with a Berkovich tip was used in order to measure the reduced elastic modulus (*Er*) and hardness (*H*) of the samples. The depth of each indentation was controlled to 100 nm and the loading, holding and unloading times were set to 5 s each. A series of ~26 and ~20 indentations, for proximal and distal sites, respectively, distributed in 3 lines, were performed at the central point of the hemi rays of the third dorsal lepidotrichia. The distance between each measurement was set 100 nm in order to minimize the effects of neighbour indentations. The load *vs.* displacement curves were plotted and the curves presenting anomalies (e.g. shoulder) were removed from the final results. In addition, both *Er* and *H* were calculated in relation to their mineral ratios obtained from the proximal regions.

## **Statistical analysis**

Descriptive analysis was performed on SEM, Raman-mapping and AFM images. All the statistical analyses were performed with SigmaPlot 12.0 (Systat Software; San Jose, CA). The normality of the samples was verified using Shapiro-Wilk Test (p < 0.01). The t-test was used to verify the statistical significance of glucocorticoid-induced osteoporosis treatment (p < 0.01). Two-way ANOVA was used for the remaining pixel-related measurements, elemental analyses, surface roughness, *Er* and *H* (p < 0.01).

# **Results**

## **Effects on mineralization by fluorescent imaging**

The pixel intensity (i.e. mineralization level) of the images captured with confocal microscopy (Fig. 1A-B) were obtained and transformed quantitatively. The values prior to and after treatment with PN were plotted (Fig. 1C). Notably, the zebrafish treated with the glucocorticoid presented a significant drop on their caudal fin bony rays’ fluorescence intensity (p < 0.01). The decreased signal intensity indicates lower mineralized tissue density, which is a characteristic of the bones with osteoporotic phenotype. The effect of medicine on the *de novo* mineralization of zebrafish’s caudal fins are shown in the various scatter plots of Fig. 1 (D-G). It is apparent that the behaviour of the regenerative process is dictated by the different conditions used (Fig. 1D-F). Also, two different cluster regions were clearly noticed in all plots: in a first phase (P1), the values were all grouped at the y-axis’ origin (RMA / RAY = 0), and in a second phase (P2), a linear increase were observed (RMA / RAY > 0).

The time-lapsed tracking of the caudal fin regeneration on non-treated subjects are displayed in the supplemented material (Supplemental Figure 2). The bright field, fluorescence and merged columns represent the caudal fin regeneration, the *de novo* mineralization and the overall regenerative process, respectively. The first mineral deposits within the regenerating tissue were stained by AR-S at 4 dpa. Fish subjects from the CTRL group displayed their first lepidotrichia bifurcation at 4 dpa.

## **Ultrastructural and compositional analysis of zebrafish bone**

A scheme of the procedures prior SEM imaging are displayed in Fig. 2A. The ultrastructural features of crushed bony fin rays are shown in the Fig. 2B. Arrangements with well-defined edges and organized in layered structures of minerals, were predominant on the proximal regions of CTRL, CTRLREGEN and ALNREGEN. The average diameter of the structures calculated for CTRL, CTRLREGEN and ALNREGEN were 102.49 (±14.64) nm, 118.63 (± 8.43) nm and 125.60 (± 10.38) nm, respectively. On distal zones, for all groups with the exception of ALNREGEN, it was observed that there was a prevalence of structures with grooves on the surface. On the other hand, for ALNREGEN distal region, structures were organized in coexistent small spherical-like and plate-like shapes. The elemental content of the SEM images was verified by EDS, and the results are shown in Table 2. The Ca content of samples in the proximal region varied from 5.77 ± 1.64 (GIOP) to 27.23 ± 3.59 (CTRL) and the P, for the same region, varied from 3.96 ± 0.50 (GIOP) to 15.68 ± 1.42 (CTRL). In all groups but GIOP, a higher percentage of Ca was found in the proximal part of the amputated fin. Similar phenomenon was observed for the Ca/P ratio; statistically significant differences was observed for CTRL, GIOP, CTRLREG and GIOPREGEN when comparing proximal to distal parts (p < 0.01). The difference in the distinct parts of ALNREGEN, however, was not significant (p > 0.01). The highest significant value was obtained for CTRL (1.74 ± 0.10) and CTRLREGEN (1.56 ± 0.06) in the proximal region (p < 0.01).

## **Calcium-phosphate profile**

Raman spectroscopy was used to analyse the intensity of the ν1 peak (~960 cm-1) in each of the zebrafish caudal fin bony rays, from proximal region to the distal tips (Fig. 3A). Three phosphate and one carbonate peaks showed to be characteristic of zebrafish appendage bones: ν1, ν2, ν4 and . The area below the ν1 peak was calculated for each of the 96 sites measured, and heatmaps representing each of the 18 lepidotrichia were produced (Fig. 3B). From proximal (A1) to distal (A5), it is clear that the ν1 intensity decreases as the distance from the resection plane increases. However, the decreased gradient of intensity was not observed in all bony rays. Moreover, CRTLREGEN and GIOPREGEN presented lower intensities at the distal parts in comparison to other groups, indicating that the newly formed bones display lower ν1intensities. ALNREGEN presented the highest area below the curve from all samples in their proximal region.

## **Surface topography and roughness**

The Figure 4A shows the methodology used to obtain the sliced samples. The 3-D surface profiles recorded for the proximal and distal caudal fin cross-sections are represented in Figure 4B. In general, the proximal regions of all groups have displayed densely-packed structures on their surfaces, with small and abrupt bumps. On the other hand, at distal locations, the previously mentioned irregular profile, was substituted by comparatively less frequent irregularities. On distal sites collagen fibrils were found emerging on the surface of a less mineralized area (Fig. 4C). The collagen fibres were found to exhibit a periodicity range from 57 nm to 94 nm (Fig. 4D). From the acquired images, the mean value of surface roughness was then calculated based on the Ra parameter; the results are described in the Supplemental Figure 4E. No statistically significant difference was observed among groups for proximal region (p > 0.01). However, the distal region of GIOP group presented significant higher roughness compared both with their proximal part and to all other distal regions evaluated (p < 0.01).

## **Mechanical properties**

The effects of PN and ALN on zebrafish caudal fin bony rays were also tested regarding the mechanical properties. Figure 5A summarizes the procedure of cross-section. The values of the *Er* (Fig. 5B) and *H* (Fig. 5C), both expressed in GPa, obtained at proximal and distal bones of the fins. By means of *Er* values, the proximal part of CTRL, GIOP and ALNREGEN were significantly higher than CTRLREGEN and GIOPREGEN (p < 0.01). If the values obtained at the proximal locations are compared within each other, the highest statistically significant value obtained was 8.68 ± 8.74 GPa (ALNREGEN) and the lowest was 4.57 ± 2.62 GPa (CTRLREGEN) (p < 0.01). By means of *H*, however, the proximal sites of both CTRL and ALNREGEN (0.29 ± 0.23 GPa and 0.34 ± 0.47 GPa, respectively), were significantly higher than the distal parts (0.06 ± 0.05 GPa and 0.02 ± 0.01 GPa, respectively) (p < 0.01). The high standard deviations found (e.g. ALNREGEN) may be related to the impossibility to perform finishing procedures on sectioned samples. Even though, statistically significant values were found. Also, both mechanical testing values were plotted in relation to the mineral ratio (Fig. 5D and 5E). The two (*Er* and *H*), despite showing a weak dependence to Ca/P (R2 equal to 0.1757 and 0.3262, respectively), showed a positive correlation.

# **Discussion**

In this work, we have implemented materials science principles to assess the properties of healthy, osteoporotic and ALN treated zebrafish bones at the multi-scale level. The fish were anaesthetized with a cocktail of Tricaine and isoflurane in order to minimize the side effects of the dopant.(32,37) The fish had their fins resected and were left to regenerate during 21 days in water tanks with or without ALN. A second resection was performed and the effects of the various treatments were tested with a series of structural, elemental and mechanical analysis. In agreement to previous research findings on higher vertebrate animal models, our study verified the reproducibility and reliability of the zebrafish model on the study of mineralized tissues. PN and ALN significantly affected the fin bones properties in comparison to the controls. Our data showed that ALN was able to restore the overall properties of zebrafish caudal fin osteoporotic bones to the levels of untreated fish. Interestingly, the bone’s appearance seems to be more affected by the region that the minerals were extracted (proximal or distal) than by the treatment proposed, while the Ca/P ratio showed difference in both situations. This reinforces the benefits of materials science approach to evaluate diseased mineralized structures in a multi-scale level. These findings provide insights in new strategies to evaluate diseased mineralized tissues, especially on those with osteoporotic phenotypes and drug action analysis. The null hypothesis was rejected.

The lepidotrichia of zebrafish is considered a powerful model to evaluate the various aspects of bone remodelling. Their ability to re-mineralize after resection is widely known.(20,24) We have thus used resection/regeneration protocols to exacerbate the drug effect before collecting the samples for analysis. By using the glucocorticoid-induced osteoporosis protocol proposed in this work, the fluorescence intensity of caudal fin bones displayed a ~15% reduction within two weeks, which configures the development of osteoporotic-like phenotype. In rodents, however, the development of osteoporosis may need increased time, depending on the bone analysed.(38) Hence, our evaluation protocol was rapid and sensitive enough to obtain significant results. Our data reconfirm the reliability of adult zebrafish caudal fin to be used as a reliable model to verify glucocorticoid-induced osteoporosis.(2)

In 2016, Cardeira *et al.*(20) suggested analysing the caudal fin *de novo* mineralization by calculating the parameters (RMA, RAY, REG and STU) of many zebrafish individuals during caudal fin regeneration. Here, the drugs’ after-effects in the lepidotrichia could be clearly observed with the methodology proposed. The scatter behaviour plotted for each treatment was strongly related to the type of medicine given to the fish. The group with no intervention during the regenerative process, had the values calculated distributed above and below the linear regression line. This may represent the natural variation of each fish when no external elements interfere in the regenerative process. Any deviation from the regression line, should indicate effects of stimulation (points above) or halt (points below) of the mineral deposition.(20) Corroborating with studies that demonstrate PN as an anti-mineralogenic compound,(15–17,39) we found lower values for the group that received no further intervention after developing osteoporotic-like phenotype (values below regression line). In contrast, most of values for fish that received the pro-mineralogenic compound (ALN), were shown above the same line (over-mineralization). Our after-effect plots suggest that this methodology may be useful for the testing different anti- and pro-mineralogenic compounds, and reconfirm PN and ALN to decrease and increase, respectively, the mineral deposition within zebrafish bones. The *in vivo* approach of time-lapsed analysis on the same subject, may have also contributed to enhance the evidence strength of our results. Considering the potential of zebrafish models, we have reconfirmed zebrafish as a suitable organism for the evaluation of bone mineralization, which could enable reduced use of rodents in future research.(22)

The *de novo* mineralization of zebrafish caudal fins involve a series of complex events.(24–26) From the initial bone formation to the development of the actinotrichia, the mineralized tissue is known to be composed of different structures, depending on the availability of Ca and P resources.(33) The intake of mineralogenic compounds could then affect the zebrafish bones at a multi-scale level.(10) We have analysed the appearance and mineral content of two distinct regions of the bony-rays affected by PN or ALN, before and after the regrowth. The mineralized structures found in the proximal region are clearly different from the distal minerals; they presented well-defined edges and contours. Similar morphologies were almost inexistent in the bones obtained from distal sites (Fig. 2B). The underlying mineralization process in the zebrafish caudal fins occurs from the proximal to the distal region by the addition of newly-formed minerals at the furthermost edge of each fin.(33) The administration of medicines did not seem to have visibly affected the appearance of the minerals observed at the proximal regions. Interestingly, the fish that received ALN displayed coexistent small rounded and plate-like shapes in the distal sites; similar structures could not be observed in high magnification SEM images in other treatment conditions. Coexisting round and plate-like minerals were found in amorphous forms of calcium-phosphates,(33,40) which are related to the first stages of mineralization.(41) The mineralization course of events starts with the saturation of an amorphous calcium phosphate (ACP) phase, followed by the mineral deposition within the collagen fibres and subsequent crystal growth.(40,42) Moreover, the bone’s natural layered structures were preserved.(34) Similar structures have been reported in previous studies.(33) Hence, our results suggest that the treatment with bisphosphonate do not reconfigure the natural morphology of bones during the 21 days of zebrafish appendage regeneration, but may have accelerated the transition between mineral development stages.

The mineral balance and bone remodelling potentials are intimately related to the Ca and P content in the site.(34) In the conditions proposed in this work, a broad range of Ca, P and mineral ratios were found in the fin’s bones. This finding corroborates with the concepts that different types of minerals coexist in zebrafish caudal fin bones.(33,40) The Ca/P proportion was found to significantly decrease in proximal-distal direction. This behaviour was observed for all but ALNREGEN group. Higher mineral ratios, reason with the less pronounced effects of mineralogenic compounds in the proximal regions. On the other hand, lower mineral availability, represented by decreased Ca/P ratios, could be more influenced by both PN and ALN.(43) This is shown by the significant lower mineral ratios found in the bones of fish after the intake of PN and the narrow difference between the proximal and distal parts of bones affected by ALN. Our multi-scale analysis suggests that ALN increase the mineral ratio of ACP in vulnerable areas first. In the same manner, PN may affect the distal areas first, which enhances the vulnerability and may favour the binding of ALN to the bone.(23)

To completely understand the effects of medicines and diseases in mineralized tissues, the evaluation of characteristics in molecular level is essential.(9,44) The intensity of the peaks observed in Raman analysis was strongly related to the vibration of the molecules excited while the test was performed.(14,44,45) Overall, the proximal regions displayed increased areas below the ~960 cm-1 peak. Our compositional analysis of Ca/P ratio has shown a great range of mineral ratios depending on the treatment and the mineral profile suggests a similar outcome. However, any difficulties related to the use of bulk characterization methods (e.g. Raman Spectroscopy) in mineralized tissues with strongly mixed crystalline and amorphous phases should not be ignored, and may also explain the results obtained.(33)

Osteoporotic-like bones are characterized by the mineral resorption caused by increased osteoclasts activity that often reconfigure bone’s microarchitecture.(3,13,46) Following the overall trend in this research, the proximal areas verified by AFM revealed a different appearance from those obtained from distal sites. The frequent and low amplitude peaks images found in the bones extracted from the proximal regions are characteristic from highly mineralized tissues.(42,47) As we analysed spots distant to the resection plane, a less mineralized characteristic was observed and collagen fibres were exposed (CTRLREGEN). The collagen fibres were presented with variable periodicity. The type I collagen commonly found in zebrafish bones is well known to have periodicity of 67 nm.(48) Lower periodicity lengths are related to the early stages of minerals in zebrafish and, in favourable conditions, the periodicity increases its length.(42) This reconfirm that the zebrafish bones are composed by different phases and development stages of Ca and P.(33,40)

The osteoporotic phenotype is related to poor mechanical properties due to its mineral deficiency and higher porosity in comparison to sound bones.(8,12) The mechanical attributes of zebrafish bones should be intimately related to the microstructure and to the composition of the mineralized tissue.(34,42,48) The highly mineralized structures found in the proximal bones showed, in general, increased *Er* and *H*. At the distal regions, where the ratio of non-mineralized tissues raises, the surfaces were found to be softer, but not significantly lower for all the conditions proposed. In proximal bones, the reduction in *H* caused by the administration of PN was countered by the intake of ALN. The efficacy of bisphosphonates is strongly related to their affinity to bind to the bones and ability to suppress the Farnesyl pyrophosphate synthase, essential to the natural pathways of osteoclasts.(10) The regulation of the balance between ossification and bone resorption led to increased mineral levels, as we demonstrated with EDS. The pro-mineralogenic compound was not only able to recover the *Er* and *H* to the original values of healthy bones, but it also showed adequate increase, not surpassing significantly the natural value of non-treated individuals.(9) Moreover, our results match with the current literature on zebrafish.(34,42,48) This is especially important since zebrafish bones are small and novel approaches are needed to assess their properties. In order to verify the relationship between the mechanical analysis and bone’s composition, we have calculated the Pearson’s correlation value. The *Er* and *H* have showed a low, but positive, interaction with the Ca/P ratios of the proximal regions affected by the medicine. This outcome reaffirms the feasibility of a multi-scale approach to study sound and diseased bones, affected or not, by mineralogenic compounds.

Our study fills a gap on the topic of diseased mineralized tissues using a time-lapsed approach of following the same individual throughout the experimentation. To the best of our knowledge, this is the first study to use a series of materials science approaches, and correlate with zebrafish bones under the effect of PN and reconfirm ALN was able to rescue the osteoporotic phenotype of the fish, which was achieved by the restoration of the *Er* and *H* to the normal range. However, although zebrafish is considered a powerful model to study bone-related disease and mineralization, there are some limitations in their use that were already summarized:(23,34) (1) The bone remodelling process differs from the mammals especially due to the presence of mono- and multinucleated osteoclasts;(23) (2) The nature of the lepidotrichia is dermal ossification,(27) which also mechanistically differs them from the mammals; (3) Despite a complete mapping of properties alongside the caudal fin bony-rays were performed and comparisons between different single pieces of bones could be done, the variability in different individuals was not tested (e.g. Raman mapping); (4) Moreover, zebrafish bones are under the effect of a different loading environment than non-aquatic mammals.(34) Thus, the results here proposed should be taken carefully. Despite that, the mechanical evaluation, in the conditions proposed, showed to be precise enough to assess zebrafish bones with different mineral levels. Our research could help to develop new strategies to evaluate overall quality of diseased mineralized tissues, especially on those with osteoporotic phenotypes and drug action analysis.

# **Conclusions**

Zebrafish caudal fin bony rays were shown to be a feasible and fast model to study the elemental, structural and mechanical characteristics of bones affected by anti- and pro-mineralogenic medicines. The administration of PN during the time proposed reduced in about 15% the fluorescence intensity of the zebrafish caudal fin bones. The anti-mineralogenic medicine also showed to reduce the Ca/P of the distal bones to 1.26, while ALN was able to partially recover it to the untreated levels (Ca/P = 1.38) and increase the mineral organisation. The mechanical behaviour of the bones was also restored to the initial range by the pro-mineralogenic compound. The presented approach may be used to test different compounds and have the potential to be used in newly developed drugs related to bone remodelling. Further research should be done regarding the long-termed intake of medicines or their dose dependence on the bone’s properties.

**Author Contribution**

F.R.B., Y-J.C., R.A. and P-Y.C. designed the study and draft the manuscript. F.R.B. was responsible for data acquisition and made figures/tables and data analysis. Y-J.C., R.A., Y-R.S. and P-Y.C. were responsible for critical comments. P-Y.C. supervised the research.

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**Table 1**: Summary of the mineralization/regeneration parameters proposed by Cardeira et al. to explore the mineralogenic performance in zebrafish caudal fin models (A); parameters used to obtain the CTRL and experimental groups proposed (B).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **A. *de novo* mineralization quantification parameters obtained by ImageJ**(20) | | | | |
| **RMA:** the real mineralized area was determined by the area inside the full contour of the mineralized rays, from the resection plane to the most distal bony rays’ tips; | | | | |
| **RAY:** the mean ray width was defined as de mean value extracted from the length of each fin ray, measured using the first segment below the resection extension; | | | | |
| **REG:** the total regenerated area was obtained from the full contour of the caudal fin tissue; | | | | |
| **STU:** the stump width corresponded to the length of the amputation plane; | | | | |
|  | | | | |
| **RMA / RAY:** Mineral deposition within the regenerated lepidotrichia; | | | | |
|  | | | | |
| **REG / STU:** Regenerated tissue within the resection plane length. | | | | |
|  | | | | |
| **B. Zebrafish treatment condition** | | | | |
| **Group** |  | **Resection** |  | **Condition** |
| **CTRL** |  | 1st |  | Untreated zebrafish |
| **GIOP** |  | 1st |  | Zebrafish treated with 125 µM PN for 14 days |
| **CTRLREGEN** |  | 2nd |  | CTRL condition + fish tank water only for 21 days |
| **GIOPREGEN** |  | 2nd |  | GIOP condition + fish tank water only for 21 days |
| **ALNREGEN** |  | 2nd |  | GIOP condition + fish tank water + 30 µM ALN for 21 days |

**Table 2**: EDS results (mean ± SD) for the proximal and distal bones extracted from zebrafish caudal fins.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Group** |  | **Elements [atomic%]** | | | | | | |  | **Ca/P ratio** | | |
|  |  | **Proximal** | | |  | **Distal** | | |  | **Proximal** |  | **Distal** |
|  |  | **Ca** |  | **P** |  | **Ca** |  | **P** |  |  |  |  |
| **CTRL** |  | 27.23 ± 3.59 |  | 15.68 ± 1.42 |  | 7.78 ± 1.22 |  | 5.35 ± 0.79 |  | 1.74 ± 0.10 Aa |  | 1.45 ± 0.05 Ab |
| **GIOP** |  | 5.77 ± 1.64 |  | 3.96 ± 0.50 |  | 6.25 ± 1.19 |  | 4.96 ± 0.92 |  | 1.46 ± 0.04 Ba |  | 1.26 ± 0.01 Bb |
| **CTRLREGEN** |  | 11.85 ± 0.76 |  | 7.60 ± 0.31 |  | 8.28 ± 0.40 |  | 6.34 ± 0.27 |  | 1.56 ± 0.06 ABa |  | 1.31 ± 0.03 ABb |
| **GIOPREGEN** |  | 10.11 ± 2.19 |  | 7.39 ± 1.51 |  | 5.28 ± 2.26 |  | 4.22 ± 1.31 |  | 1.37 ± 0.02 Ba |  | 1.25 ± 0.21 Bb |
| **ALNREGEN** |  | 11.57 ± 1.41 |  | 7.78 ± 0.83 |  | 4.46 ± 0.81 |  | 3.24 ± 0.67 |  | 1.49 ± 0.03 Ba |  | 1.38 ± 0.04 ABa |

Capital letters mean significant statistical difference in the same column (p < 0.01).

Small letters mean significant difference in the same row (p < 0.01).

**Figure 1**: **Glucocorticoid-induced osteoporosis development** **and** s**catter plots showing the *de novo* mineralization *vs.* the overall regeneration**. Lepidotrichia fluorescence signal before **(A)** and after **(B)** the treatment with PN. Representation of fluorescence intensities for all the fish analysed **(C)**. Healthy fish regenerating in fish tank water only **(D)**; fish with osteoporosis phenotype regenerating in fish tank water only **(E)**; fish with osteoporosis phenotype regenerating in fish tank water containing ALN **(F)**; all treatments plotted in the same graphic with linear regression analysis showing the strong relationship between the variables **(G)**. The P1 and P2 (linear increase) regions are displayed in “D” separated by the dashed line. R2 showed the strong and positive interaction between variables. Different capital letters indicate statistically significant difference (p < 0.01).

**Figure 2**: **Scheme and** **SEM images of proximal and distal crushed bones extracted from zebrafish caudal fins before and after the proposed treatments, in ×20k and ×100k magnifications**. Processes performed prior SEM analysis **(A)**. Proximal crushed parts showed minerals with bigger size and well-defined shapes, which was not commonly found in distal counterparts (white arrowheads), while the distal sites showed minerals resembling amorphous phases (yellow arrowheads). The distal region of ALNREGEN showed grouped small spherical-like (light blue arrowheads) and plate-like morphologies (red arrowheads) **(B)**.

**Figure** **3**: **Montage containing a representative zebrafish caudal fin bony ray and the Raman spectra obtained.** Representation of the 17th lepidotrichia (dorsal region of the fin) of the ALNREGEN group isolated from the fluorescence images obtained in confocal microscope **(A)**; five different spots of interest along the growth direction were measured **(A1-5)** and the respective Raman peaks were obtained for each region. Heat colour plots representing the total area below the curve for the phosphate peak of ~962 cm-1; the black arrow indicates the bony ray represented in the Raman spectra shifts above **(B)**. The legend box shows the total values of the areas divided by 103. The darker the spots, the higher the mineral intensity of the signal.

**Figure 4**: **Surface topography profile of zebrafish bony rays and roughness calculation**. Procedure steps from the resection of the zebrafish caudal fins, to the analysis with AFM in tapping-mode **(A)**. 3-D representation of the cross-section slices (obtained by cryosection process) analysed by AFM **(B)**. The distal part of CTRLREGEN group showing collagen fibres bundles (white single arrows) in flattened representations **(C)**. Approximation of the collagen fibre bundle from the white square in “B” **(D)**; the double arrows shows the diameter of a periodic unit from a fibril (d ~ 75 nm). Box-plots with means (red horizontal line) and median (black horizontal line) of the roughness obtained from proximal and distal parts of fins by AFM **(E)**. Capital letters indicate significant statistically difference between the different regions (proximal and distal) within the same treatment (p < 0.01). Lowercase letters indicate significant statistically difference between the same region (proximal or distal) among different treatments (p < 0.01).

**Figure 5: Mechanical performance of zebrafish caudal fin bony rays.** Procedure steps to obtain the caudal fin’s slices for mechanical evaluation **(A)**. Box-plots with means (red horizontal line) and median (black horizontal line) of the *Er* **(B)** and *H* **(C)** obtained from proximal and distal parts of fins with a tribo-indenter. Capital letters indicate significant statistically difference between the different regions (proximal and distal) within the same treatment (p < 0.01). Lowercase letters indicate significant statistically difference between the same region (proximal or distal) among different treatments (p < 0.01). The average values of the proximal parts of both *Er* **(D)** and *H* **(E)** plotted against the Ca/P ratio. The regression lines show a weak but positive correlation between the variables.

**Supplementary Figures**

**Supplementary Figure 1: Flow-chart with the experimental protocols used in this study.**

**Supplementary Figure 2**: **Bone and tissue regeneration process of zebrafish caudal fin**. The bright field column represents the tissue growth; the fluorescence column represents the *de novo* mineralization process; the merged column images represent the overall process (tissue + mineral) regeneration. The scale bar sizes are 500 µm.

Figure 1



Figure 2

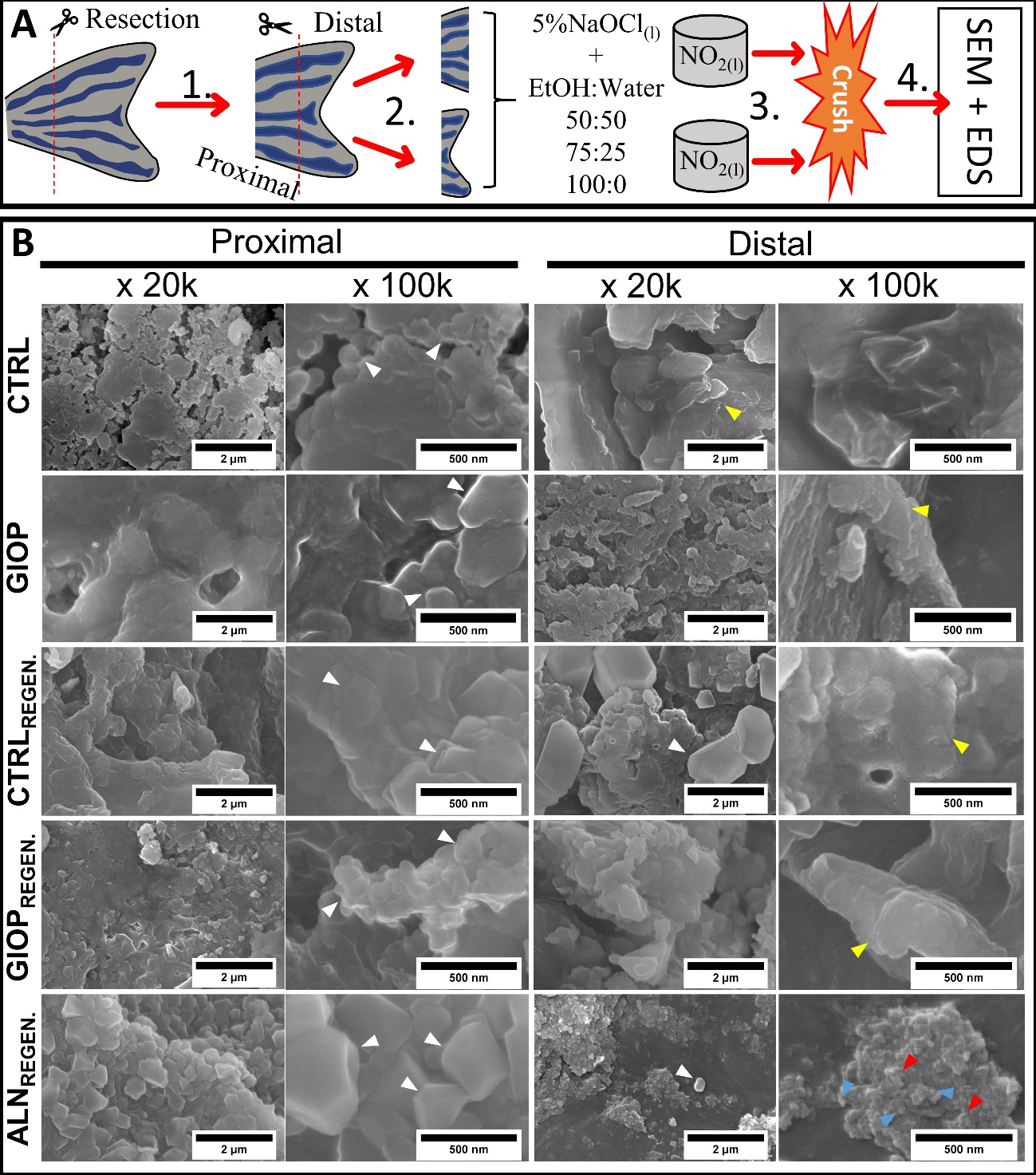


Figure 3



Figure 4



Figure 5



Supplemental figure 1



Supplemental figure 2

