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ORIGINAL ARTICLE

Alendronate rescued osteoporotic phenotype in a model of glucocorticoid-induced osteoporosis in adult zebrafish scale

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SUMMARY

Long-term effects of glucocorticoid treatment in humans induce bone loss and increase the risk of fracture in the skeleton. The pathogenic mechanisms of glucocorticoid-induced osteoporosis (GIOP) are still unclear. The GIOP and its effects have been reproduced in several animal models including *Danio rerio* (zebrafish) embryo. The treatment of adult fish with prednisolone (PN) has shown a dose-dependent decrease of mineralized matrix in the scales. Large resorption lacunae are characterized by single TRAP-positive cells which migrate to the margin of the scale merging into a multinucleated structures. The treatment with PN of cultured scales did not increase TRAP activity suggesting that the massive presence of osteoclasts in the resorption sites could be likely the result of a systemic recruitment of monocyte–macrophage precursors. We observed that treatment with PN induced a significant decrease of the alkaline phosphatase (ALP) activity in scale scleroblasts if compared with untreated controls. Then, we investigated the total mineral balance under prednisolone treatment using a time-dependent double live staining. The untreated fish fully repaired the resorption lacuna induced by prednisolone, whereas treated fish failed. The presence of osteoclast resorption fingerprints on new matrix suggested that the osteoclast activity counterbalances the osteodepositive activity exerted by scleroblasts. The treatment with PN in association with alendronate (AL) has surprisingly resulted in a significant decrease of TRAP activity and increase of ALP compared to PN-treated fish in biochemical and histological assays confirming the action of alendronate against GIOP in fish as well in humans.

Keywords
bone matrix, glucocorticoid-induced osteoporosis, scale, zebrafish

Introduction

Glucocorticoids (GC) are employed in the therapy of many chronic inflammatory disorders such as arthritis, Crohn's disease, ulcerative colitis and pulmonary diseases. The long-term use of glucocorticoids causes several adverse side effects including a loss of bone able to determine an increasing risk of fracture in the skeleton. A specific syndrome has been classified owing to the symptomatic and evident effects of long-term GC treatment in humans, called glucocorticoid-induced osteoporosis (GIOP) (Maricic 2011). The pathogenic mechanisms of GIOP are still unclear. It has been reported that patients treated with GC show an early, rapid increase in bone resorption followed by a chronic decrease in bone deposition (Maricic 2011).

However, it was documented that GC interferes with osteoblast differentiation and behaviour *in vitro* and *in vivo* (Chavassieux *et al.* 1993; Weinstein *et al.* 1998).

In contrast, the effects of glucocorticoids on osteoclasts are debated. Some data reported that osteoclastogenesis is increased in mice after 7 days of treatment with prednisolone, whereas it was decreased by 60% after 21 days (Weinstein *et al.* 1998). An additional study in mice lacking glucocorticoid receptor by osteoclasts demonstrated that glucocorticoids prolonged the life span of osteoclasts (Kim *et al.* 2006). In RANKL-knockin mice, a 4-week

prednisolone treatment increased the number of differentiated osteoclasts (Hofbauer *et al.* 2009). The GIOP and its effects have been reproduced in animal models for studying the mechanisms underlying the bone metabolism imbalance induced by these hormones when used within pharmacological doses.

In mouse model of GIOP, long-term administration of glucocorticoids induces a osteoporotic phenotype (Weinstein *et al.* 1998). Similar effects have been demonstrated in additional, different animal models such as beagles (Quarles 1992), ewes (Chavassieux *et al.* 1997), rats (Lindgren *et al.* 1983), rabbits (Baofeng *et al.* 2010) and pigs (Scholz-Ahrens *et al.* 2007) with different times of onset.

In the last decades, *Danio rerio* (zebrafish) has been affirmed as a powerful animal model to study bone development given the high degree of similarity of both bone architecture and genetics with those seen in humans.

The zebrafish embryo treated with prednisolone, proposed by Fleming *et al.* as model of GIOP, has shown a significant delay in early mineralization process (Barrett *et al.* 2006).

Whereas embryonic osteogenesis is easily studied in zebrafish embryos, the adult zebrafish represents an excellent model to investigate bone metabolism and remodelling. Indeed, osteoblasts and osteoclasts are simultaneously activated inducing the regulation of adult bone mass as well as is seen in adult humans (Witten PE *et al.*, 2009).

The body surface of the teleost fish is covered by calcified structures named scales. Anatomically, the zebrafish scale is a flat plate organized in two overlapped layers. The external one, which represents the episquamal side, is constituted by calcium phosphate and hydroxyapatite crystals in a structure which is very similar to the human woven bone (Sire *et al.* 2004). The scale-forming cell is named scleroblast and exhibits a strong functional similarity with human osteoblast (Metz *et al.* 2012). Episquamal scleroblasts are organized in a single layer and are responsible for the deposition of the mineralized matrix during scale remodelling, whereas few resident osteoclasts drive the remodelling-associated resorption (de Vrieze *et al.* 2011). The internal layer, also named the basal the plate, which represents the hyposquamal side, is composed of calcified collagen type I matrix organized in a plywood-like arrangement. Hyposquamal scleroblasts, as well placed in monolayer, depose and organize the collagen fibrils (Sire & Akimenko 2004; de Vrieze *et al.* 2011). The fish scale has been recently assessed as model to measure the activity of scleroblasts and osteoclasts *in vivo* after pharmacological treatments (Suzuki *et al.* 2000, 2008, 2009; de Vrieze *et al.* 2014a,b).

Recently a GIOP model has been published where in zebrafish where regenerating scales shown an osteoporotic-like phenotype after treatment with prednisolone (de Vrieze *et al.* 2014a,b).

In the present work, we propose a model of GIOP in adult zebrafish using non-regenerating scale as read-out system to evaluate the effects of prednisolone and/or alendronate on bone metabolism of naïve scales.

Materials and methods

Animals and scale collection

The Danio rerio AB strain was maintained routinely in a ZebTec Bench top (Tecniplast, Italy) under standard condition at 28°C. Six month old male zebrafish of similar weight and length were treated for 2 weeks through directly immersion in a E3 medium solution containing prednisolone 50 μM (Sigma, Italy), alendronate 30 μM (Sigma) or both. To collect scales fish were anaesthetized in 0.01% tricaine methanesulphonate (Sigma) and scales were carefully removed uniformly from the anterior area of either side of the body under a dissecting stereomicroscope (Wild M3Z, Heerbrugg, Switzerland) using Dumont® Stainless Steel Forceps (Sigma). When necessary fish were euthanized using a 0.1% tricaine methanesulphonate.

Ethics statement

All experimentation in the Zebrafish Lab (IRCCS R. Galeazzi, Milan, Italy) conforms to the ITA and EU guidelines on research practice (European directory 2007/526/CE). The zebrafish experimentation is authorized by ASL Varese with Prot. No. 014AVB0020033, Italy.

Detection and quantification of mineralization

Bone matrix of explanted scales was stained with 0.2% calcein dissolved in aqueous solution. The calcium content of fish scales was evaluated indirectly through a new method performed in our laboratory. Explanted scales were fixed with 3.4% formaldehyde/0.1 M Sodium Phosphate Buffer solution and stained for 1 h at room temperature with Alizarin red dissolved in a 1% KOH solution to a final concentration of 10 μg/ml. After aspiration of the unincorporated dye the scales were washed and incubated overnight with a 20-mM TRIS buffer containing 0.25 M EDTA pH 8.00 as extraction agent. Coloured supernatant was transferred in 96-well plates, and the absorbance was evaluated at 450 nm as indirect value of scale calcium content on microplate reader (TECAN GENios Plus).

Biochemical and histological TRAP activity

The optimal conditions to assess tartrate-resistant acid phosphatase (TRAP) activity by biochemical assay have been described by Persson *et al.* (1995). After being fixed, the cultured scale was incubated in 0.1 M sodium acetate buffer (pH 5.3) containing 20 mM tartrate. After 1 h, the medium was replaced with a substrate solution composed of 20 mM pNPP (4-nitrophenyl phosphate disodium salt hexahydrate; Sigma) and 20 mM tartrate dissolved in a 0.1 M sodium acetate buffer. The reaction was stopped by adding 2 N NaOH solution, and the amount of p-nitrophenol produced was spectrophotometrically assayed at 405 nm (Fusion™ Universal Microplate Analyzer; Packard, USA) transferring

the coloured supernatant in a 96-well plates. The absorbance was converted into the amount of produced para-nitrophenol (pNP) using a standard curve for pNP. Histological demonstration of TRAP activity on osteoclastic scale cells was performed with an acid phosphatase detection kit (Sigma, procedure no. 387) according to the manufacturer's protocol.

Biochemical and histological ALP activity

Specific osteoblast alkaline phosphatase (ALP) activity was biochemically assayed according to our method described previously (Pasqualetti *et al.* 2012a,b) with a 450-μl alkaline buffer (100 mM TRIS–HCl, pH 9.5, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$) containing 20 mM pNPP for 1 h. The reaction was stopped with 3 N NaOH and 20 mM EDTA solution, and the absorbance of the supernatant evaluated at 405 nm transferring the coloured solution in a 24-well plates. The absorbance was converted into the amount of produced para-nitrophenol (pNP) using a standard curve for pNP.

Alkaline phosphatase positive scleroblasts were histologically detected using BCIP/NBT ready-to-use solution as a liquid substrate (Sigma, Italy) according with the manufacturer's protocol.

Bone matrix vital staining

Evaluation of the bone matrix profile of the explanted scales from prednisolone treated fish was performed through successive treatments with vital staining solutions containing Alizarin red (Sigma, Italy) or Calcein (Sigma) respectively, in accordance with previously published papers (Kimmel *et al.* 2010). Briefly, 20 fish were treated with prednisolone for 14 days than vitally stained with 0.005% Alizarin red S overnight in the dark. Next, a group of 10 fish was incubated for additional 14 days in a complete E3 medium while the second experimental group of 10 fish was still exposed to prednisolone for additional 14 days. At the end of the treatment, a second pulse was performed on both animals using 0.005% calcein solution overnight. After repeated washes with fish water, scales were collected from anaesthetized fish, fixed with 3.4% formaldehyde 0.1 M Sodium Phosphate Buffer solution and analysed using a fluorescence microscope.

Scanning Electron Microscopy (SEM) analysis

Scales collected from control and prednisolone treated fish were fixed with 0.125 M sodium cacodylate buffered solution containing glutaraldehyde (Sigma) and paraformaldehyde (Sigma, Italy) in a final concentration of 0.25% and 0.2% respectively. Fixed scales were washed with a tamponed 0.1 M sodium cacodylate solution and macerated following the osmic maceration method (Congiu *et al.* 2004) through immersion in a aqueous solution of 1% OsO_4 (Società Italiana Chimici) and 1.25% potassium ferrocyanide (Sigma) 2 h at room temperature.

After osmium maceration scales were dehydrated in ascending grades of ethanol for 10 min at each passage and 20 min twice in absolute ethanol, subjected to chemical drying incubating for 30 min in a 100% hexamethyldisilazane solution (Fluka, Italy) according to standard procedures for SEM processing and air-dried at room temperature for 3 h. Subsequently, dried scales were placed on stubs with conducting tape and gold coated using a sputter coater (*Emitech K-550 sputter-coater*). High-energy beam of electrons was performed with a scanning electron microscope (Philips XL30 SEM-FEG FEI Company, Eindhoven, Netherlands) both in secondary electron image (SE) mode and in backscattered electron image (BSE) mode.

Scale culture

In accordance with our method recently described (Pasqualetti *et al.* 2012a,b), explanted scales from AB zebrafish were cultured in fresh Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, L-glutamine and 2% penicillin-streptomycin mixture in a CO_2 incubator (Euroclone, Italy) at 28°C under 5% CO_2. In the treated group, prednisolone was added to a final concentration of 50 μM. At the end of the treatment, each group of scales was washed with phosphate-buffered saline (PBS), transferred as single scale per well in a 96-well microplate and processed for the evaluation of TRAP activity at three different time points, 24, 48, 72 h according with the method previously described and at 96 h to confirm the decrease of cells viability (Pasqualetti *et al.* 2012a,b). The medium was changed every 24 h.

Nuclear staining

Fluorescence nuclear staining was performed on fixed scales incubated for 15 min with 0.1 μg/ml 4′,6-diamino-2-phenylindole (DAPI; Sigma, Italy).

Microscope analysis

Stained scales were placed on a glass slide, soaked with glycerol and mounted with coverslips for microscope analysis. Images were obtained using Olympus SZX-ZB7 fluorescence stereomicroscope (Olympus, Italy) equipped with a Blacklight 5000 camera (Tiesselab, Italy).

Statistical analysis

Statistical significance was determined for *P*-values being set at <0.05*; $P < 0.01$**; $P < 0.001$*** using the Student's *t*-test or, in case of treatment with glucocorticoids and bisphosphonate, by one-way analysis of variance with multiple comparison with the Bonferroni method. All the experiments were repeated at least three times with comparable results. In different experimental conditions, calcium content was quantified in 50 fish scales; TRAP and ALP enzymatic activity were quantified in 20 scales. Results are shown as the mean ± standard deviation or percentage different *vs.* controls.

Results

Prednisolone reduces scale mineralized matrix in adult fish

It is known from the literature that in humans and higher vertebrates administration of GC leads to osteoporosis characterized by a loss of bone mineral matrix (Manelli & Giustina 2000). We examined whether lower vertebrate model-like fish could reproduce the osteopaenic condition. To highlight the possible effects of glucocorticoid on mineralized matrix, we used adult zebrafish treated with prednisolone (PN) and we evaluated the amount of mineralized matrix in the scales.

Experimentally 10 adult zebrafish were treated for 15 days with PN in decreasing concentrations: 50, 5 and 0.5 μM administered directly into the water, subsequently changed every 24 h. Another group of 10 fish without treatment was considered as a control group. After treatment, the fishes were sacrificed and 100 scales per animal were incubated with alizarin red. Thus, the dye was extracted by treatment with EDTA and read at 560 nm. The quantitative analysis has detected a dose-dependent decrease of mineralized matrix in scales treated with PN compared to controls, (Figure 1a) although not statistically significant.

Then we investigated the presence of areas of matrix resorption. We performed the experiment analysing by fluorescence microscopy the explanted scales stained with calcein. In scales of fish treated with PN the presence of a fringed margin located mostly along the anterior/lateral part of the scale was found (Figure 1b) suggesting that the resorption lacunae may be restricted to the scale edge.

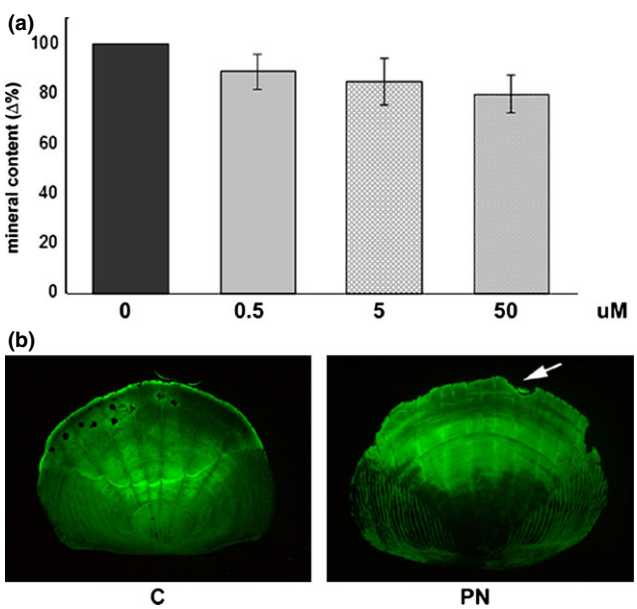

Figure 1 Loss of bone matrix after PN treatment. (a) Dose-dependent decrease of mineralized matrix in scales treated with 0, 0.5, 5 and 50 μM PN. (b) Fluorescence image after calcein staining of explanted scale from untreated and PN-treated fish. A loss of mineralized matrix is shown along the anterior/lateral edge of the scale.

Prednisolone enhances osteoclast resorption activity in the zebrafish scales

It is known from the literature that the decrease of the mineralized matrix in the GIOP may depend on an increase in the activity of osteoclasts and/or by a decrease of the activity of osteoblasts (Mazziotti *et al.* 2006). The presence of resorption lacunae in the scales of fish treated with PN suggests that the osteoclasts activity could be involved.

To test whether a decrease in the mineral content could be due to an increased activity of osteoclastic cells, we evaluated the activity of a typical marker of bone resorption such as tartrate-resistant acid phosphatase (TRAP) in the scales.

Experimentally 10 adult zebrafish were exposed for 15 days to 50 μM PN and a group of 10 untreated fish were considered as a control. After treatment, the fish were sacrificed and 100 scales per animal were explanted and used to assess TRAP activity by biochemical assay. Quantitative analysis for TRAP revealed an increase in the enzymatic activity in scales of fish treated with PN (Figure 2a). The enzymatic TRAP analysis of a 100 pooled scales does not describe the behaviour of individual scales in response to glucocorticoids. Therefore, the TRAP enzymatic assay was carried out on 10 individual scales treated with PN and 10 untreated controls and values reported in Figure 2b. The analysis on single scales confirmed the data obtained on pool.

The enzyme TRAP is a marker of osteoclast activity that carries out its hydrolytic action when released in the microenvironment of the resorption lacuna. Given the presence of the resorption lacunae in scales treated with prednisolone, we analysed the distribution of the osteoclast activity in the scale by an histological TRAP staining. The experiment was performed as the previous biochemical assay by changing the TRAP substrate (see *Materials and methods*). In the scales treated with PN, we observed a strong TRAP staining along the anterior/lateral edge (Figure 2c).

The control and the PN-treated scales were further analysed with the scanning electron microscope (SEM) to best investigate the morphological characteristics of the resorption surface (Figure 2d).

Non-resident osteoclasts are recruited in the scale by prednisolone

It has been demonstrated clearly that the bone loss after treatment with GC is mediated by the recruitment of osteoclast precursors from the blood circulation in the resorption site (Lindgren *et al.* 1983).

First, we aimed to studying the real time needed to stimulate the osteoclast-dependent resorption activity by PN in zebrafish scale.

Experimentally 10 adult zebrafish were incubated for 2, 4, 7 and 15 days with 50 μM PN. A different group of 10

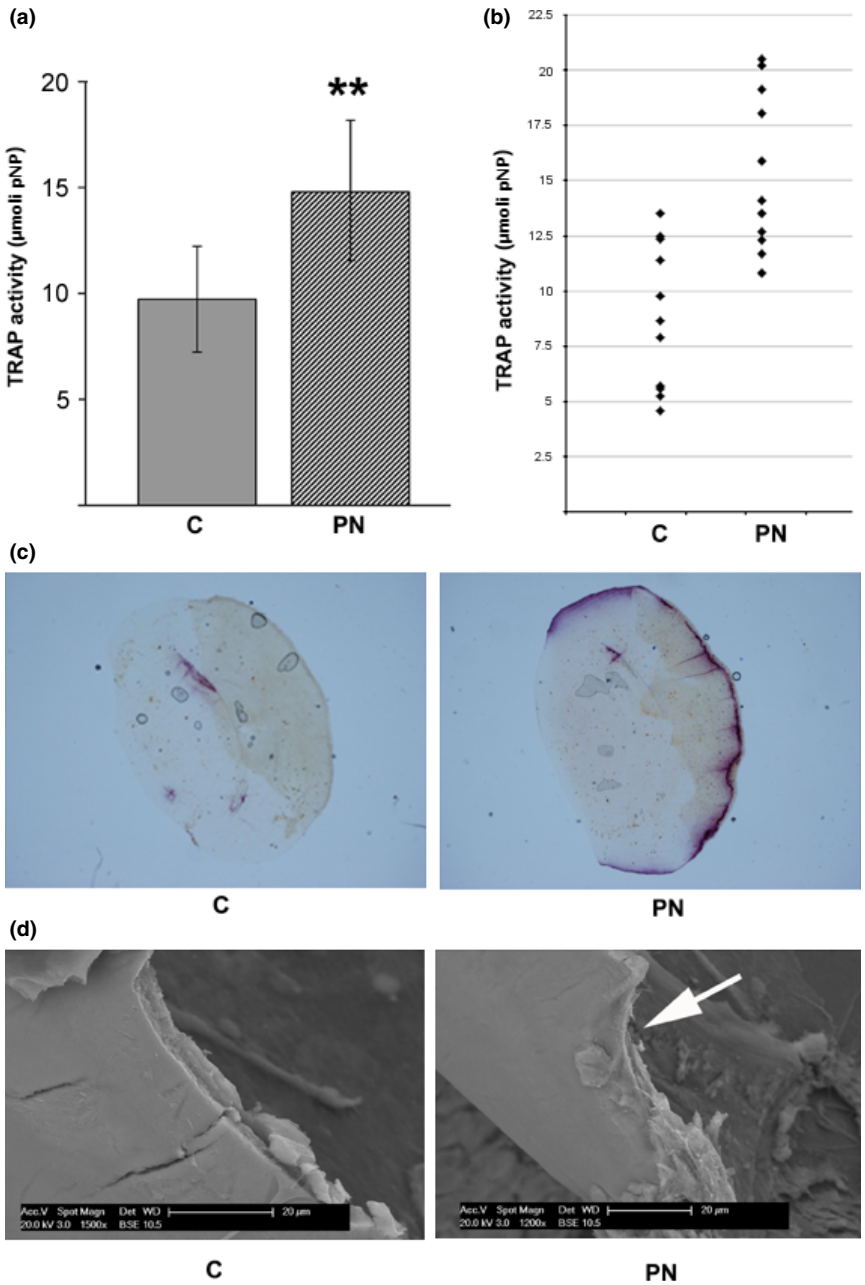

Figure 2 TRAP activity in zebrafish scales treated with PN. Biochemical assay for TRAP activity in pool (a) and in single scale (b). Quantitative analysis revealed an increase in the enzymatic TRAP activity in scales of fish treated with PN. (c) TRAP staining of scales treated with PN indicated a resorption activity along the anterior/lateral edge. (d) scanning electron microscope (SEM) imaging of the edge of scale treated or not with PN. Resorption patterns are clearly distinguishable in PN-treated scales.

fish was considered as untreated controls. After treatment, the fish were sacrificed and 100 scales per animal were explanted and used to assess the activity of TRAP enzyme by histochemical assay. Some traces of resorption activity have been detected already at day 2, while at day 4 and 7, the signal becomes evident reaching the maximum at 15 days (Figure 3a).

After two weeks of treatment, the high-magnification images shown that the resorption site is characterized by the formation of a TRAP-positive syncytium localized along the edge of the scale. Single TRAP-positive cells are clearly visible close to the resorption site (Figure 3b).

Given the high number of osteoclasts involved, we subsequently investigated whether the activated osteoclastic cells in the resorption site were derived from a in-scale resident population or from out-scale circulating precursors. Scales of untreated fish were explanted and cultured in a 96-well plate with D-MEM 10% FCS containing PN 50 µM at 28°C for 24, 48 and 72 h. After incubation, for each time points, the scales were fixed and processed for biochemical TRAP assay.

Treatment of cultured scales with PN did not result in a significant increase in TRAP activity compared to the controls (Figure 3c) suggesting that the massive presence of osteoclasts in the resorption sites could be likely the result of a systemic recruitment of monocyte–macrophage precursors in agreement with what was observed in the pathogenesis of GIOP in humans.

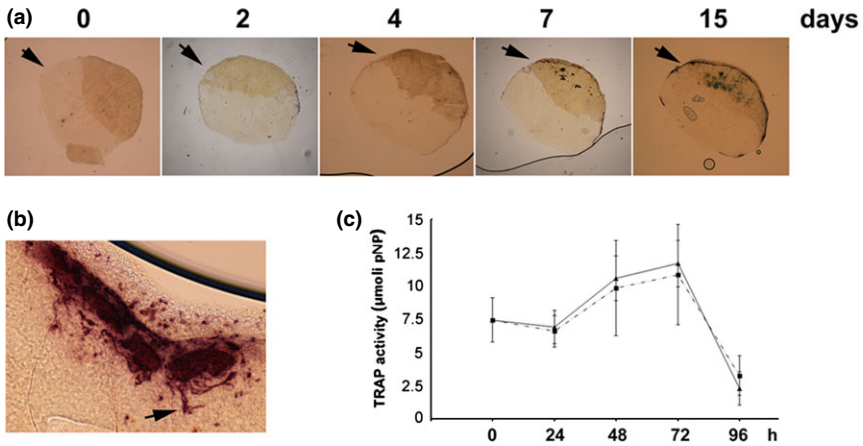

Figure 3 Osteoclasts activation pattern in presence of PN. (a) Histochemical TRAP assay on scales from fish treated with PN for 2, 4, 7 and 15 days. A weak signal of resorption activity has been detected from the second day of treatment with 50 μM PN. (b) high-magnification (40×) image of histochemical TRAP staining showing the osteoclastic syncytium and single TRAP-positive cells migrating towards the resorption site. (c) Biochemical TRAP assay on cultured scales. No significant increase in TRAP activity has been reported after PN treatment (■) with respect to controls (▲).

Prednisolone inhibits ALP activity

A decrease of the bone deposition rate has been shown to play a role in the pathogenesis of GIOP due to a direct action of GC on osteoblastic cells as demonstrated by a reduction of ALP activity (Chavassieux *et al.* 1993).

We therefore decided to investigate whether the treatment with PN was able to induce a decrease in the ALP activity and/or a reduction of the number of scleroblasts in zebrafish scales.

Experimentally, 10 adult zebrafish were incubated for 15 days with PN 50 μM. Another group of 10 untreated fish were considered as a control. After treatment, the fish were sacrificed and 100 scales per animal were explanted. The scales were pooled and used to assess the activity of ALP by biochemical assay. We observed that treatment with PN is able to induce a significant decrease of the ALP activity in scales if compared with untreated controls (Figure 4a).

To investigate whether the reduction of ALP was due to a loss of scleroblastic cells or a modulation of the enzymatic activity, we performed an histochemical ALP activity assay in combination with a nuclear DAPI staining. We histologically evaluate the single-cell ALP activity in the available scleroblasts of the scale explanted from fish treated or not with PN and the eventually presence of cell's mortality. The presence of several ALP-negative cells after the incubation with PN (Figure 4b) suggests that the glucocorticoid is able to inhibit the ALP activity in zebrafish scale scleroblasts without affecting their vitality as demonstrated by their positivity on DAPI staining.

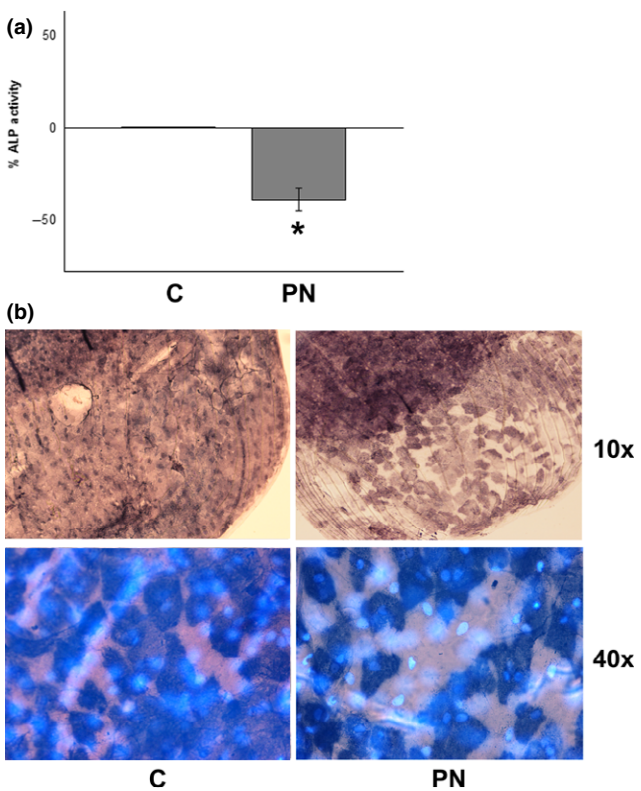

Figure 4 ALP activity in zebrafish scales treated with PN. (a) Biochemical assay for ALP activity. PN administration induced a significant decrease of the ALP activity in scales if compared with untreated controls. (b) Histochemical ALP activity assay and DAPI staining. The presence of several ALP-negative cells was found after the incubation with PN. Magnification: upper panel 20×, lower panel 40×.

Bone matrix deposition balance is impaired in scales treated with prednisolone

However, the ultimate effect of PN on bone tissue is determined through the evaluation of the balance between deposition and resorption of the mineralized bone matrix. First of all, we wanted to verify whether after induction of a PN-dependent osteoporotic phenotype, the scleroblasts were

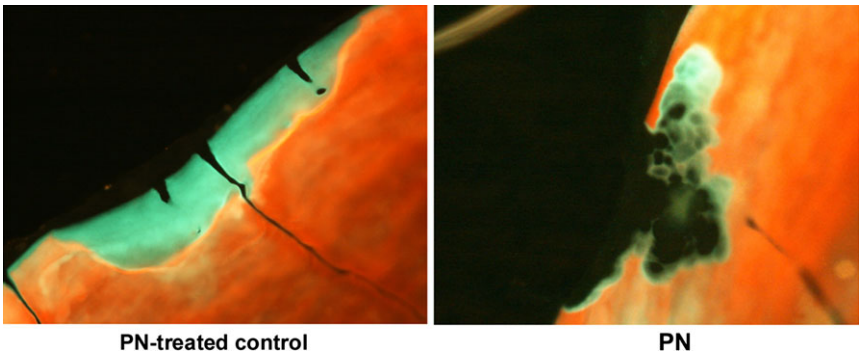

Figure 5 Mineralization defects induced by prednisolone. The fish were treated with PN and, at the end, vitally stained with Alizarin red (red). Next, the fish were treated or not with PN and successively stained with Calcein (green) to evidentiate the new mineralized matrix. The untreated fish fully repaired the resorption lacuna, whereas PN-treated fish failed.

still functional and able to restore the resorbed bone. 20 adult zebrafish were treated for 14 days with PN to induce a osteoporotic phenotype. Live staining with Alizarin red was performed to mark the resorption lacunae. Next, 10 fish were kept incubated with PN, whereas 10 control fish were incubated in fish water for additional 7 days. At the end of the treatment, all the fish were stained with calcein as evidence of the mineralized matrix specifically deposited in the last 7 days. As shown in Figure 5, the scale scleroblasts of control fish have fully repaired the resorption lacuna by filling the lacunae with new mineralized matrix (green signal), whereas scale scleroblasts from PN-treated fish have failed to repair the PN induced damage. The presence of osteoclast resorption fingerprints on new matrix suggested that the osteoclast activity counterbalances the osteodepositive activity exerted by scleroblasts.

Alendronate suppress prednisolone-dependent bone loss

It is very well documented the use of bisphosphonates in the therapy for GIOP (Saag *et al.* 1998). Several studies demonstrated the ability of this class of molecules to inhibit osteoclasts activity in response of GC treatment (Rogers 2004) while the direct effects of bisphosphonates on osteoblastic cells are still under debate.

In order to validate our model of GIOP, we decided to treat the fish with prednisolone and alendronate (AL) and evaluate whether the action of bisphosphonates is able to counteract the osteoporotic effects induced by GC also in zebrafish.

Ten adult zebrafish were incubated for 15 days with PN 50 μM, AL 30 μM or both. Another group of 10 untreated fish were considered as a control. After treatment, the fish were sacrificed and 100 scales per animal were explanted

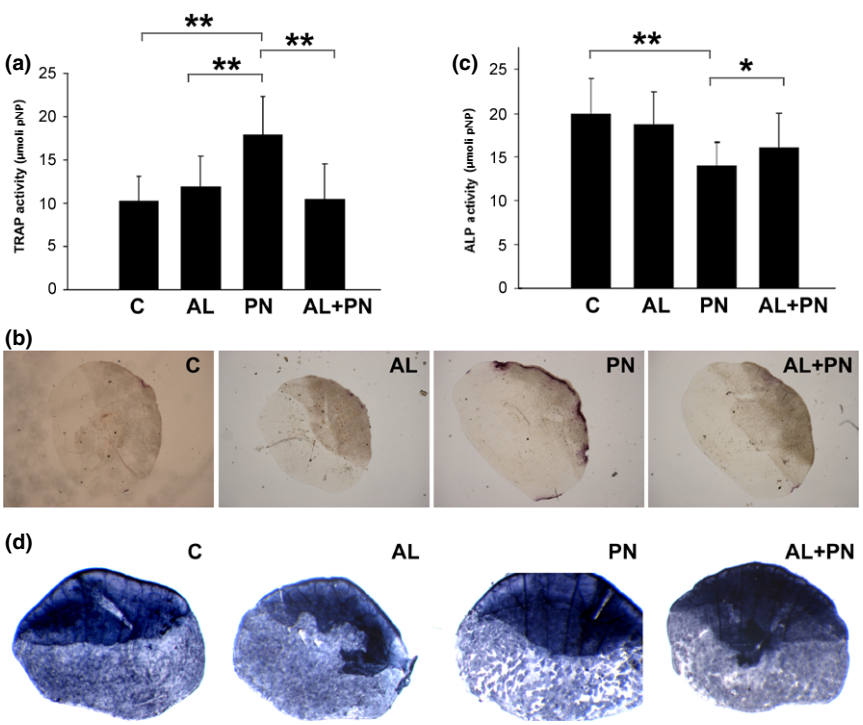

Figure 6 Alendronate suppress prednisolone-dependent bone loss. Biochemical (a) and histological (b) assay for TRAP activity on scales treated with PN, AL or AL+PN. This data demonstrated that bisphosphonates are able to inhibit the bone resorption induced by GC. Biochemical (c) and histological (d) assay for ALP activity on scales treated with PN, AL or AL+PN. The treatment with AL is able to inhibit the negative effects induced by the PN on osteoblast ALP activity.

and analysed for TRAP and ALP activity through biochemical and histological assays.

The treatment with PN in association with AL has surprisingly resulted in a significant decrease of TRAP activity compared to PN-treated fish in biochemical assay (Figure 6a). The histological analysis showed that the double treatment with AL and PN decreased the extension of the TRAP-positive resorption sites at the edge of the scale (Figure 6b). In both experiments, the TRAP activity after treatment with PN was comparable with the previous tests as the only treatment with AL does not cause effects on the activity of osteoclastic cells. This data demonstrated that bisphosphonates are able to inhibit the bone resorption induced by GC.

The biochemical detection of ALP activity has demonstrated how the treatment with AL is able to inhibit the negative effects induced by the PN on scleroblasts (Figure 6c). The histological analysis showed that the treatment with AL maintained the ALP activity similar to the controls in scale scleroblasts (Figure 6d).

Taking together, these results confirm the action of alendronate against GIOP in fish as well in humans, suggesting highly conserved molecular mechanisms between two species.

Discussion

The pathogenesis of GIOP is still largely unknown, but several *in vitro* and *in vivo* studies have been addressed to clarify the molecular basis of the disease. As well noted the *in vitro* approach is limited because the use of cell cultures treated with GC does not take in consideration the crosstalk between different type of cells and tissues in a whole body context. Several animal models have been proposed as a model of GIOP, in which a long-term administration of GC induces a osteoporotic phenotype. Nevertheless, the experimental variability in timing and method of administration, bone density evaluation method, makes difficult to reach a definitive explanation of the pathogenesis.

A simple animal model with high conservation in bone metabolism regulation systems could help to increase the knowledge about the pathogenesis of GIOP and prove useful as *in vivo* pathological model to test new drugs against the disease.

The zebrafish embryo treated with prednisolone has been already proposed as model of GIOP. Nevertheless, this model is limited because the regulation systems involved in the early embryonic osteogenesis are very different from those involved in adult bone metabolism. In addition, the osteoclasts, when present, do not play the same role in adult and during the developmental stage.

We proposed the adult zebrafish as an innovative model of GIOP because it is very similar to humans in terms of bone anatomy and metabolism and because it is easy to evaluate different bone parameters using the scale as readout system.

The biochemical quantitative analysis has detected a trend of loss of mineralized matrix in scales explanted from fish treated with PN and the calcein staining showed the presence of the characteristic resorption lacunae along the posterior margin of the scale. The presence of localized resorption lacunae similar to those observed in remodelling human bone has confirmed that osteoclast activity could play a fundamental role.

The increase of the TRAP activity in the scales treated with PN and the strong TRAP staining of the resorption lacunae indicates that osteoclasts are massively activated by PN.

Physiologically, the presence of basal osteoclast activity in the scale is discontinuous and localized mainly in the grooves, whereas the posterior margin does not require resorption activity during osteodeposition. The total number of TRAP-positive scales increase suggests that each scale is able to respond to PN switching-on the bone matrix resorption.

In addition, the formation of an extensive resorbing surface, often observed in human bone tissue, rarely occurs during normal bone remodelling in fish, and our data demonstrate that it is highly stimulated by PN. The consequence of the high level osteoclast activity has been corroborated by three-dimensional SEM images where it is notable how the osteoclasts massively reabsorb the mineral component of the episquamal calcified thin layer. The scale culture represents an elegant experiment to demonstrate such recruitment because the scale can be considered as explanted organ isolated from the blood circulation. The results have demonstrated that the osteoclast involved in the resorption is not derived from a resident cell population since at 72 h of treatment no activated osteoclasts can be detected. We can hypothesize that, like in humans, the osteoclasts responsible for intense bone resorption are recruited from monocyte lineage present in the blood circulation. The presence of single TRAP-positive cells that converge in the resorption site fusing each other supports the similarity of this pathway between humans and fish.

Together these data suggest that the treatment with PN activates the osteoclasts through an alternative pathway in which a non-resident population of osteoclastic precursors is massively recruited from the blood circulation to the scale where they immediately begin to degrade the bone matrix.

It is known that the effect of GC on osteoclasts is conducted at different levels. First, the GC promote the expression of RANK-L by osteoblasts, thereby increasing the differentiation and activation of osteoclasts, while reducing the expression of the soluble RANKL receptor, osteoprotegerin (OPG) (Hofbauer *et al.* 1999). Dexamethasone promotes the expression of the protein macrophage colony-stimulating factor (M-CSF) by the osteoblastic cells that in the presence of RANK-L stimulates the process of osteoclastogenesis (Rubin *et al.* 1998). Homologous forms of RANK, RANKL and OPG have been found in fish suggesting a conservation of the crosstalk pathway between osteoblast and osteoclast (Kitamura *et al.* 2013). In our GIOP model,

GC could stimulate active osteoblasts to produce RANKL which, in turn, stimulate the recruited osteoclastic precursors to differentiate *in loco*. The high conservation of the crosstalk pathways between osteoblast and osteoclast underlines the relevance of this animal model in the study of human bone resorption processes.

It has been shown that GC have a direct action on human osteoblastic cells reducing ALP activity and osteodeposition rate (Lindgren *et al.* 1983). We observed also in zebrafish that treatment with PN induced a significant decrease of the ALP activity in scales without changes in the number of scleroblasts. As it has been proposed that scleroblastic ALP promotes bone mineralization, our data suggest the role of PN to negatively modulate scale's scleroblasts resulting in a defective mineralization rate during bone deposition in the fish scales.

Nevertheless, the effects of PN on scleroblast and osteoclasts have been clearly demonstrated in the present work and the real effect of PN on bone tissue should be demonstrated by the analysis of the mineralized matrix. The zebrafish scale represents an excellent model to monitor the metabolism of the mineral tissues by multiple live staining. Live incubation with calcein and alizarin red at different times allows to visualize the time-dependent mineralization of the bone tissue.

Our experiments demonstrated that, after discontinuation of PN treatment, the scleroblasts are able to fully repair the bone lacunae together with the intermission of the resorption activity. On the other hand, scleroblasts under continued PN treatment have failed to repair the lacuna. The presence of small amount of new mineral matrix along the resorption front demonstrated that the scleroblasts are activated to repair the lacuna. Nevertheless, as the new matrix showed evident traces of osteoclast fingerprints, we suggested that the bone demolition activity counterbalances the bone deposition one, resulting in the prevention of the lacunae healing. In our hypothesis, the PN-mediated osteoclast activation is responsible for the massive resorption in the scale. Next, the scleroblasts become activated to fill the gap, but they are not able to counteract the huge resorption activity, also because they are less efficient in presence of PN. The surprising action of osteodeposition by scleroblast even in the presence of PN may be due to a osteoclast-driven stimulation. It is known that the presence of osteoclasts, independently from their resorbing activity, is needed for normal bone formation (Pederson *et al.* 2008).

The use of bisphosphonates in the therapy for GIOP has been demonstrated to counteract the activation of the osteoclasts mediated by GC. We demonstrated that, also in zebrafish, the treatment with alendronate, in association with PN, decreased the TRAP activity, if compared with PN-treated fish, and prevents the loss of ALP activity. The protective effects of alendronate in zebrafish GIOP model suggest that the preservation of the pharmacological response reflects the preservation of bone metabolism pathways between human and fish. For these characteristics, the zebrafish GIOP model can be used in drug screening experiments to select new potential drugs against human GIOP. The zebrafish has several advantages in comparison with different animal models: early onset of the osteoporotic phenotype, small size, administration method and low costs of stabulation. In particular, the use of the scale, as read-out system, permits one to analyse the behaviour of bone cells and the mineral matrix remodelling with high efficiency due to a low invasivity, high number of samples, easy handling and imaging, live staining and low cost.

These results underline the ultimate utility of using fish (and scales) as model to assess the effects of drugs on bone tissues.

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Conflict of interest

It is declared that the named authors on this manuscript have no conflicts of interest regarding the contents of the manuscript.

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