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Original Article

Resveratrol Protects against Rankl-Induced Mineralized Bone Damage in a Transgenic Medaka Fish Model of Osteoporosis

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Abstract: Natural substances with bone protective potential always greatly attract researchers for the development of safer and more effective drugs for osteopenia and osteoporosis. Resveratrol (RES), a plant polyphenol found in red grapes and berries has been commonly suggested as supplement for bone health. It has shown bone anabolic and anti-resorptive effects in different *in vitro* cell cultures and *in vivo* rodent models. Recently, the medaka fish *(Oryzias latipes)* has emerged as a valuable model organism for bone research. In this study, we present, for the first time, evidence that resveratrol, tested at five doses (25, 50, 100, 150, and 200 μM), mitigated bone loss induced by Rankl in a transgenic medaka model for osteoporosis. Notably, the dose of 150 μM exhibited the highest bone-protective and anti-resorptive effect, reducing bone loss by approximately 20%. However, at the same dose, RES did not significantly affect the signal densities of green fluorescent protein (GFP) and alizarin complexone (ALC), which represent osteoblasts and mineralized matrix, respectively, in *col10a1*:nlGFP transgenic medaka larvae. In these larvae, osteoblasts were marked by GFP, and bone was stained with ALC. Our findings provide significant evidence from a non-rodent model regarding the therapeutic potential of resveratrol.

Keywords: Medaka, osteoporosis, rankl:HSE:CFP, resveratrol, rankl.

1. Introduction

Bone health is maintained by the balance between bone formation by osteoblasts (bone-forming cells) and bone resorption by osteoclasts (bone "eating" cells). Bone resorption outpacing bone formation can reduce bone mass and bone disorders, including osteoporosis [1].

Osteoporosis is a common human bone disease featured by reduced bone mass, deteriorated bone structures, and a high risk of bone fragility. The disease presents a significant

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global public health challenge, owing to the increasing incidence among aging populations and the prevalence of unhealthy lifestyles [1].

RANKL (Receptor activator of nuclear factor kappa-B ligand) is a key stimulator for osteoclastogenesis, the formation and differentiation of osteoclasts. Overexpression of RANKL therefore can result in increased bone resorption and osteoporosis. RANKL and RANKL/RANK signaling pathways are potential targets for the development of drugs for osteoporosis [2].

Medications for osteoporosis can be classified as either anti-resorptive or bone anabolic agents, which respectively inhibit bone resorption or increase bone formation. Traditional anti-resorptive drugs, such as bisphosphonates (e.g., alendronate and etidronate), are associated with high costs, limited efficacy, and adverse effects [1]. Thus, research to develop more efficacious and safer therapeutic interventions for osteoporosis has increased, of which the search for antiosteoporitic agents from natural resources is of great interest.

Resveratrol is a natural polyphenolic compound that is commonly found in red grapes, berries and medicinal plants such as *Fallopia multiflora (Hà thủ ô đỏ)*. The compound has been shown various health benefits including antioxidants, and anticancer [3-5]. Resveratrol has been suggested as a food supplement for bone health [6]. There have been numerous studies in different *in vitro* cell cultures and *in vivo* rodent models and a clinical trial showing bone anabolic and anti-resorptive effects of resveratrol [6-11]. The FDA's guidelines (1994) recommend the use of non-rodent animal models alongside rodent models for evaluating the therapeutic potential of agents [12]. In this study, we investigated the bone-protective effect of resveratrol for the first time in medaka fish.

Medaka (*Oryzias latipes) (*and also zebrafish *(Danio rerio))* has been emerged as a preferable model organism to study human diseases and to develop drugs [13-16]. These fish own biological features that facilitate experiments such as short generation time (2-3 months), high fecundity, external fertilization, external development of transparent embryos, and low cost for maintenance [14-17]. Thus, transgenesis and mutagenesis can be performed [18-20] and biological processes at cellular and molecular levels can be observed in the fish with ease using fluorescence imaging techniques [21-24]. Numerous transgenic and mutant medaka fish have been generated to study the mechanism of bone metabolism and bone diseases [21-23, 25]. Among those, the *rankl:*HSE:CFP medaka fish has been created to model Rankl-induced osteoporosis [23]. Osteoporosis-like phenotype in form of demineralized bone structures can be induced in this fish larvae upon a heat-shock at 39 ^{o}C and a CFP (Cyan blue fluorescence protein) signal is used as an indicator for this transgenic fish. The fish was brought to our laboratory and since has been further segregated into sublines that show different levels of mineralized bone damage [26]. The c8 subline, whose osteoporosis phenotype is mostly visible in mineralized neural arches, was used for this study. In this fish subline, the level of bone damage can be assessed using the index of bone mineralization (I_M) - the total lengths of 15 first mineralized neural arches [27]. We have used this fish subline to evaluate the bone protective effects of some natural substances. Icariin and oleanoic acid (OA) have been shown to reduce bone damage in the neural arches of this Rankl fish [27, 28].

The data obtained from this study will contribute to the growing evidence regarding the bone-protective potential of resveratrol.

2. Experimental

2.1. Reseratrol Source

Resveratrol with a purity of 99.8% as determined by HPLC was isolated and purified at the National Institute of Medical Materials from the root of *Fallopia multiflora* collected in Vietnam as published by Phuong Thien Thuong et al., [29].

2.2. Fish Lines and Fish Maintenance

In this study, two transgenic fish including the *rankl*:HSE:CFP subline c8, which was segregated in our laboratory [26] from the original *rankl*:HSE:CFP fish provided by National University of Singapore [23, 26, 27], the *col10a1*:nlGFP fish [30], and wild-type fish were used. The subline c8 of the *rankl*:HSE:CFP fish (hereafter named as Rankl fish/embryos/larvae) exhibits osteoporosis phenotype in form of damage in mineralized neural arches [26, 27]. Experiments were performed on hemizygous Rankl embryos/larvae offspring of homozygous Rankl and wild-type fish parents. The *col10a1*:nlGFP fish is an osteoblast reporter line which expresses green fluorescent protein (nlGFP) in the nuclear of *collagen10a1* expressing osteoblasts [30]. Fish were cultured and maintained as previously described, at temperature of 28–30 ^{o}C with 14 hour light: 10 hour dark cycle [26, 27]. Rankl fish embryos were screened by fluorescent reporter CFP at 11 days post fertilization (dpf), 2 days after heat-shock [27]. All fish experiments were performed in accordance with the animal welfare laws and guidelines from Dinh Tien Hoang Institute of Medicine, Hanoi, Vietnam (Approval number: IRB-AR.002)

2.3. Heatshock for Generation of Osteoporosis Phenotype in Rankl Fish Embryos

Rankl fish embryos at age of 9 dpf were heat shocked at 39 ^{o}C for 90 minutes to generate osteoporosis like phenotype as previously reported [23, 26, 27].

2.4. Real-time Quantitative PCR

11 dpf WT and c8 fish samples were RNA isolated with TRIzol and purified with DNaseI (Themofisher); then these RNA samples were used to synthesize cDNA according to the Themofisher method. The synthesized cDNA samples were stored at -20 ^{o}C and used for real-time qPCR reactions with master mix SYBER GREEN (Themofisher) on an AB 7500 machine to obtain C_t values. From these Cts, the expression level of the *rankl* gene between the WT and the c8 fish group was analyzed and compared according to the $2^{-(\Delta\Delta CT)}$ method of Livak [31]. The primers used for the *rankl* gene are 5'-CCATCGAGGAGGTCCAGG- 3' (forward primer) and 5'-CCG TTC CGA TAG GAC ATT CGG-3' (reverse primer), which amplify a DNA fragment of 197 bp.

The primers used for β-actin gene are 5'-GcAGCGCCGTCACACACAGC-3' (forward primer) and 5'-GGATACTTCAGGGTCAGGATACC-3' (reverse primer), which amplify a DNA fragment of 297 bp (Phusa genomics).

2.5. Resveratrol Treatment

RES treatment was carried out following procedures reported previously [27]. As RES is soluble in DMSO, a RES stock solution of 100 mM was prepared in anhydrous DMSO. This stock solution was further diluted in E3 solution to final concentrations or tested doses of 25 μM, 50 μM, 100 μM, 150 μM, and 200 μM (final DMSO concentration was 0.4%). Seven days post fertilization (dpf) Rankl hemizygous larvae were divided randomly into groups and raised in a medium containing RES at a tested dose (+Rankl +RES groups) or containing 0.4% DMSO (DMSO control or +Rankl-RES group) or containing alendronate at the dose of 25 μg/ml (positive control or + Rankl + Alen group) in a well of a 24-well cell culture plate. Rankl larvae were heat-shocked at 9 dpf at 39 ^{o}C for 90 minutes. After a 2-hour recovery period at 30 ^{o}C, the drug-containing medium was changed, and embryos were raised to 11 dpf when they were screened to confirm CFP expression before being fixed with 4% paraformaldehyde (PFA) for alizarin red bone staining. Experiments were repeated until each group contained a statistically sufficient number of fish (greater than 30 fish per group). A group of heat-shocked wild-type embryos (Wt group) were also included as a control for bone development without drug treatments.

2.6. Staining of Mineralized Bone Structures and Imaging

To visualize the mineralized bone matrix of 11 dpf fish larvae, the fish were fixed and

stained with alizarin red (Sigma A5533) as previously described [27].

2.7. Imaging

Imaging for fixed and live bone-stained fish larvae was performed as described previously [23, 27].

Fixed, alizarin red bone-stained fish were imaged using an Axiovert 100M microscope (Carl Zeiss, Germany). For live fish with fluorescence signal, fish larvae were anesthetized with 0.01% ethyl 3-aminobenzoate methanesulfonate (tricaine; Sigma A5040) and embedded in 1.5% low-melting-point agarose in a glass-bottom petri dish. Images were acquired using a 10× EC Plan-Neofluar N.A. 0.3 objective on an Axio Observer 7 fluorescent microscope equipped with Apotome 2 (Carl Zeiss) for both green (green fluorescent protein – GFP) and red (alizarin complexone – ALC) channels. For each embryo, 32–40 optical slices covering a 117–146 μm thick section were collected, but only 32 optical slices were used to generate a 3D total intensity projection (Zen software, Carl Zeiss). The quantification of GFP and ALC signal intensities was performed on 2D images, in a region of interest (ROI) covering five (6^{th}–10^{th}) vertebrae using ImageJ software (NIH).

2.7. Quantification of the Level of Bone Mineralization and Bone Mineralization Damage

I_M method [27] was used to assess the level of bone mineralization and level of bone mineralization damage of fish larvae via Index of bone mineralization (I_M) and Index of mineralization damage (I_D), respectively. I_M is the sum of lengths of the first 15 mineralized neural arches ($I_M = \sum_{k=1}^{15} L$, where k is the ordinal number of neural arch and L is the length of each arch; I_M is indicated in pixel (px)). Based on the I_M of Rankl fish and of WT fish, I_D of Rankl fish was calculated by the formular: $I_D = [I_{M\ (WT)} - I_{M\ (Rankl)}]/I_{M\ (WT)}$ x 100%, where I_D is the percentage of mineralization damage of neural arches of a larva, $I_{M\ (WT)}$ is the Index of bone mineralization of wild-type fish, and $I_{M\ (Rankl)}$ is Index of bone mineralization of the corresponding Rankl fish. RES at doses that have significantly higher I_M values than that of DMSO control is considered to have antiresorptive and bone protective effect in this fish model; The index of mineralization protection I_P of each dose was calculated by the formular: $I_P = (I_{M\ (+Rankl\ +RES)} - I_{M\ (+Rankl\ -RES)})/ I_{M\ (-Rankl-RES)}$.

2.8. Statistical Analysis

"Student t-tests (two-tailed, unequal variance) or one-way ANOVA followed by Tukey's multiple comparison test were used for comparing groups and determining significance, using Prism 5 (GraphPad Software Inc., San Diego, CA). Differences with a P value less than 0.05 were considered significant. Results are presented as mean ± S.E.M.

3. Results and Discussion

3.1. Results

3.1.1. Level of Mineralized Bone Damage in the Rankl Fish used for the Study

To ensure that the subline c8 of the *rankl*:HSE:CFP transgenic fish (the Rankl fish) own suitable osteoporosis-like phenotype for this study, we checked for the expression of the transgene *rankl* and the level of mineralized bone damage in fish larvae. As expected, real-time quantitative PCR showed that the expression of the transgene *rankl* in heat-shocked Rankl fish is about 17.62 folds higher than in wild-type fish (Figure 1a). This indicates that the transgene *rankl* was still highly expressed in the transgenic fish at the mRNA level.

Alizarin red staining revealed damage in the mineralized neural arches of 11 dpf Rankl fish larvae (white arrow heads, Figure 1b), 2 days after being heat-shocked at 39 ^oC for 90 minutes, compared to intact bone structures of wild-type control fish (black arrowhead, Figure 1b). Quantitative assessment using I_M method results

in an I_M value of 834.4 (pixel) for Rankl fish and of 3487.4 (pixel) for WT fish. Thus, the Index of mineralization damage I_D of these Rankl fish was calculated as 76.1% (see 2.7 for the formula to calculate I_D). This is a level of bone damage suitable for further experiments in this study.

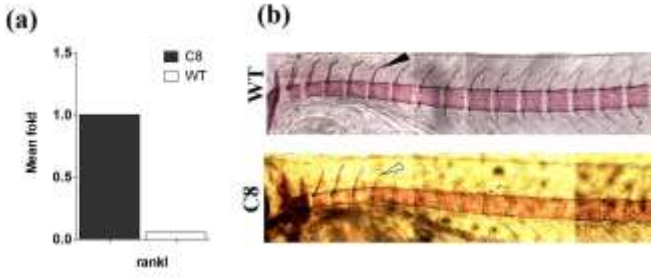

Figure 1. rankl expression and mineralized bone phenotype of Rankl compared to wild-type fish.
(a) expression level of rankl gene in Rankl fish (C8) and wild-type fish (WT).
(b) representative images of mineralized bone of the first 15 vertebrae stained with 0.4% alizarin red of 11 dpf Rankl (C8) and WT fish subjected to heat shock at 39 ºC for 1.5 hours. Black arrowhead indicates an intact neural arch in the WT fish group; White arrowhead indicates a damaged neural arch in the Rankl fish group.

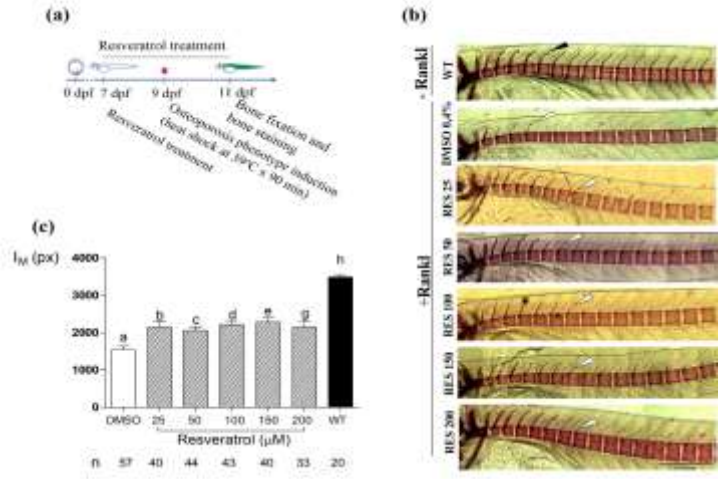

Figure 2. Resveratrol reduces mineralized bone damage in Rankl induced fish larvae.
(a) Experimental procedure to evaluate the effect of Resveratrol on bone damage of Rankl induced fish. The red arrow indicates heat-shock induction, green fish is Rankl-induced**.**
(b) Representative images of bones of the first 15 vertebrae stained with alizarin red 0.4% of 11-day-old fish groups in the experiment to evaluate the effects of Resveratrol: wild-type fish (WT;-Rankl), Rankl induced (Rankl+) DMSO control group (DMSO 0,4%), Rankl induced (Rankl+) fish groups treated with resveratrol (RES) at concentrations of 25, 50, 100, 150, 200µM. Black arrowhead indicates intact neural arch bones in wild-type fish groups; White arrowheads indicate damaged neural arch bones in +Rankl fish groups.
(c) Mean values of bone mineralization index I_M of fish groups in the experiment. a – h: I_M values of Rankl induced control fish group treated with 0.4% DMSO; with resveratrol 25, 50,100, 150, 200 µM and WT were respectively 1531.5;2147.6; 2059.7; 2210.4; 2286.4; 2146.5; 3489.7; px: pixels. a≠c ($p<0.05$); a≠ d,e,h ($p<0.001$);a≠ b,g ($p<0.01$); a,b,c,d,e,g≠h ($p<0.001$) (Differences between values were determined by ANOVA test and Turkey post-test). n: number of fish in corresponding groups. The bars at the top of each column represent the S.E.M.

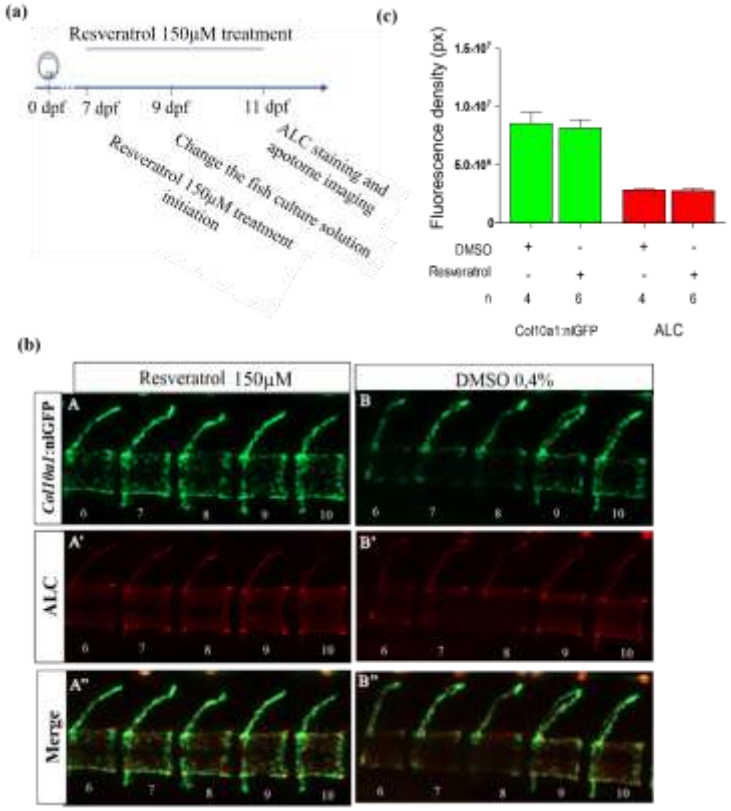

Figure 3. Resveratrol does not affect osteoblasts and mineralization levels of col10a1:nlGFP fish.
(a). Experimental procedure to evaluate the effect of Resveratrol on osteoblast cell density.
(b). Fluorescence images of 5 vertebrae (segments 6-10) of representative fish of the Resveratrol treatment group (A-A") and control fish (B-B"); Image of a z-stack layer with a thickness of 117 µm). A, B: Green GFP fluorescence signal (col10a1:nlGFP) expressed under the control of the collagen10a1 gene promoter in osteoblastic cells. A',B' alizarin complexone (ALC) red fluorescence signals of mineralized vertebral bones. A": merged image of two GFP (A) and ALC (A') signals of Resveratrol-treated fish; B": merged image of two GFP (B) and ALC (B') signals of control fish.
(c). Statistical analysis of the density of GFP green fluorescence signals expressed by collagen10a1-expressing osteoblasts and ALC red fluorescence signals measured in control and Resveratrol-treated fish. +/- indicates fish treated/not treated with Resveratrol; n: number of fish analyzed per group. Two-tailed, unpaired t-test. $p>0.05$. Bars indicate S.E.M. Scale bar: 0.06 mm.

3.1.2. Resveratrol Reduced Mineralized Bone Damage in the Rankl Fish Model for Osteoporosis

To check for the ability of resveratrol (RES) to protect bone from damage induced by Rankl in the fish, RES was used at doses of 25, 50, 100, 150, and 200 µM to pretreat and treat Rankl-induced fish larvae as described in "RES treatment" and depicted in Figure 2a. At 11dpf, experimental fish larvae were fixed and stained with alizarin red for bone mineralized structures. Images of 15 first vertebrae of bone-stained larvae were taken, and the level of bone damage of fish larvae was assessed via I_M method. Results are shown in Figure 2b, c. As seen in Figure 2b, while wild-type larvae have an intact bone matrix of neural arches (black arrowheads in Figure 2b), all Rankl-induced fish larvae (including larvae treated with 0.4% DMSO as control (DMSO control (+Rankl+DMSO)); and with RES (+Rankl+ RES+DMSO) show different extents of

damages in mineralized neural arches (white arrow heads in Figure 2b). However, the extents of bone damage of all RES-treated fish seem less than those of DMSO control fish (Figure 2c). Quantitative assessment of levels of mineralization by I_M method confirmed the observation. All RES-treated fish groups have significantly higher mean I_M values than that of DMSO control group **(**RES 100 µM (2210.35, n=43), RES 150 (2286, n=44) vs. DMSO (1531.5, n=57) ($p<0.001$); RES 25 (2147.58, n=40), RES 200 (2146.54, n=33) vs. DMSO ($p<0.01$); RES 50 (2059.7, n=44) vs. DMSO ($p<0.05$) (Figure 2.c). Thus, RES of all tested doses can protect mineralized neural arches of heat-shocked Rankl fish from damage induced by Rankl. The protection Indexes (I_{Ps}) of RES at these doses are calculated (see for formular for I_P calculation) as 16.9%, 14.5%, 18.6%, 20.7%, and 16.9%, respectively. Thus, RES shows anti-resorptive effect in the Rankl-induced fish and RES at the dose of 150 µM has the highest effect.

3.1.3. RES has no Significant Effect on Col10a1- Expressing Osteoblasts and Mineralization of Fish Larvae

To evaluate the effect of resveratrol (RES) on mineralized bone matrix and osteoblasts, RES at 150 µM was used to treat transgenic *col10a1*:nlGFP fish whose osteoblast progenitors expressing *collagen10a1 (col10a1)* gene were marked by green fluorescence signal. *col10a1*:nlGFP embryos were maintained in medium containing RES of 150 µM from 7 to 11 dpf and the medium was changed at 9 dpf. Control embryos were raised in medium with 0.4% DMSO. Fish larvae were stained with alizarin complexone to visualize mineralized matrix in red, and *col10a1* positive osteoblasts were observed by green fluorescence at 11 dpf (Figure 3(a)). Thus, GFP and ALC fluorescence signal densities indirectly reflect osteoblast expression and level of mineralization of fish (Figure 3 (a)).

Osteoblasts and mineralization of a larva were representatively analyzed on 5 vertebrae (from the vertebrae 6 to the vertebrae 10) through GFP and ALC fluorescence signal density. Representative Apotome images and fluorescence densities of the two fish groups of the experiment are shown in Figures 3(b), (c). The results showed no difference in the density of both GFP and ALC fluorescence signals between the two groups of resveratrol-treated fish and the control fish (GFP: 8.6×10^6, n=4 vs. 17×10^6, n= 6, $p > 0.05$; ALC: 2.9×10^6, n=4 vs 2.7×10^6, n= 6, $p>0.05$ (Figure 3 (b); (c)). This shows that resveratrol at 150 µM does not affect the expression level of *collagen10a1* gene and pro-osteoblasts expressing *collagen10a1* nor affect the level of bone mineralization of fish.

3.2. Discussion

Resveratrol (3, 5,4'- trijhydroxystilbene) (RES) is a natural substance belonging to the family of non-flavonoid polyphenic compounds found abundantly in *Fallopia multiflora*, red grapes and some foods like red wine and chocolate. It has shown a wide range of potential health benefits including antioxidant, anti-aging, anti-cancer properties, cardiovascular and bone protection [3-5].

The effects of RES on bone have been demonstrated by a variety of studies using different research models, ranging from *in vitro* [6-8], *in vivo* [5, 7, 9, 10], to a human -clinical trial [11]. RES has been shown to stimulate osteoblast differentiation [6] inhibit osteoclast formation [5, 7, 10] both *in vitro* and *in vivo*. It was also able to increase bone mass and density in ovariectomized rats [7]. Very recently, a meta-analysis of Zhao et al. on 15 studies on the effect of RES on osteoporosis-induced rats have shown that RES may increase bone mineral density in osteoporosis rat models through improving bone microstructure and regulating calcium and phosphorus metabolism [32].

There still be only one clinical trial pilot program to evaluate the effect of RES in reducing age-related osteoporosis (RESHAW) was conducted in postmenopausal women. Supplementation with 75 mg of RES twice daily for 12 months had a positive effect, increasing bone density in the lumbar spine and femoral neck [11].

The bone-protective effects of RES appear to be very promising. The purpose of this study was to evaluate the bone protective effect of RES on a Rankl-induced osteoporosis medaka fish model, a new non-rodent model to provide more complete evidence for therapeutic potential of this compound. Our results show that RES at all five tested doses ranging from 25 to 200 µM could protect mineralized bone matrix by significantly reducing Rankl-induced bone damage. The substance achieved bone protection levels ranging from 14.5-20,7%, nearly equivalent to that of alendronate, a common anti-resorptive drug at the dose of 25 µg/ml [27]. It is worth noting that the differences in the effect of RES between the studied doses were not statistically significant, suggesting that RES did not exhibit a dose-dependent effect in the tested dose range of this study as reported by previous studies using *in vitro* model and *vivo* models [6].

RES is known to have low toxicity, and cell-level studies have shown that RES inhibits osteoclast differentiation and function at a concentration range of 0.3-10 µM [10] or 12.5-100 µM [32]. The dose range for fish studies is usually chosen as approximately 10 times higher than that studied in cells [27]. We chose the above-mentioned dose range for this study on fish due to the wide dose range used in cell studies. It is possible that the dose-dependent effect of RES on the Rankl-induced fish model can be observed with doses outside this dose range. Nevertheless, as Rankl is a key stimulator for osteoclastogenesis, the fact that RES could reduce bone damage induced by Rankl in this fish model suggesting the ability of the substance to inhibit the formation and function of Rankl-induced osteoclasts, as evidenced by previous studies [7, 10, 33].

Our study also initially investigated the effect of RES at 150 µM on the bone mineralized matrix and osteoblasts in *col10a1*:nlGFP fish which express GFP in collagen10a1-expressing osteoblast precursors. Comparative analysis of GFP green and ALC red fluorescence signal intensity in five representative vertebrae between the RES-treated and control groups revealed no statistically significant difference. This indicates that RES at 150 µM had no significant effect on *collagen10a1* gene expression or on the number and density of osteoblast progenitors expressing this gene [30]. Additionally, RES did not impact the mineralization level of fish bones, as indirectly expressed through the ALC red fluorescence signal in this experimental design. These results diverge from those of previous studies using cell or rodent models, which have shown that RES can stimulate the differentiation and function of osteoblasts, increasing osteogenic activity and bone density [5-9]. However, further studies may be needed to evaluate the bone anabolic effect of RES at higher doses, possibly through the expression of other osteoblast-specific genes such as twist [34], osterix [35] osteocalcin [36], or alkaline phosphatase [37].

4. Conclusion

Resveratrol mitigated bone damage induced by Rankl in an osteoporosis medaka fish model, offering protection for approximately 20% of the mineralized bone matrix, despite exhibiting no significant effect on osteoblasts or the mineralized bone matrix. These findings contribute valuable evidence from a non-rodent model regarding the therapeutic potential of resveratrol.

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