



Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

journal homepage: www.elsevier.com/locate/jphs

Full Paper

Liquiritigenin reduces osteoclast activity in zebrafish model of glucocorticoid-induced osteoporosis

Marta Carnovali ^a, Giuseppe Banfi ^{a, c}, Massimo Mariotti ^{a, b, *}^a IRCCS Orthopedic Institute Galeazzi, Via R. Galeazzi 4, 20161, Milan, Italy^b Department of Biomedical, Surgical and Dental Sciences, University of Milan, Via Commenda 10, 20122, Milan, Italy^c Vita-Salute San Raffaele University, Via Olgettina 58, 20132, Milan, Italy

ARTICLE INFO

Article history:

Received 1 April 2020

Received in revised form

11 May 2020

Accepted 28 May 2020

Available online 3 June 2020

Keywords:

Zebrafish

Liquiritigenin

Osteoporosis

Bone metabolism

Scales

ABSTRACT

Drug and therapies currently used to treat human bone diseases have a lot of severe side effects. Liquiritigenin is a flavonoid extracted from *Glycyrrhiza glabra* roots which has been reported to have positive effects *in vitro* on osteoblasts activity and bone mineralization as well as inhibitory effect on osteoclasts differentiation and activity *in vitro*. The present study was aimed to evaluate the *in vivo* effects of liquiritigenin on bone structure and metabolism in physiological and pathological conditions using *Danio rerio* as experimental animal model. Treatments with liquiritigenin were performed on embryos to evaluate the osteogenesis during skeletal development. Other treatments were performed on adult fish affected by glucocorticoid-induced osteoporosis to assay the therapeutic potential of liquiritigenin in the reversion of bone-loss phenotype in scale model. Liquiritigenin treatment of zebrafish embryo significantly enhances the osteogenesis during development in a dose-dependent manner. In addition, liquiritigenin inhibits the formation of the osteoporotic phenotype in adult zebrafish model of glucocorticoid-induced osteoporosis preventing osteoclast activation in scales. Interestingly, liquiritigenin does not counteract the loss of osteoblastic activity in scales. The liquiritigenin exhibits *in vivo* anti-osteoporotic activity on adult fish scale model. It can be considered a good candidate to develop new drugs against osteoporosis.

© 2020 The Authors. Production and hosting by Elsevier B.V. on behalf of Japanese Pharmacological Society. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Dietary intake of vegetables and fruits has an important impact on bone health. For this reason, great attention is given to the identification of natural substances capable of modulating bone metabolism and mineral density including polyphenols and in particular flavonoids.^{1,2} The drugs currently used to treat human bone diseases have shown many important side effects³ so it is crucial to identify innovative non-invasive natural therapeutic approaches. To this purpose, herbal extracts represent a potential source of new molecules active on bone metabolism. Indeed, nutraceuticals and herbal extracts have been used for centuries in traditional medicine and numerous studies show how these

molecules can play an important role in the prevention of tissue degeneration in many human skeletal diseases because they naturally possess anti-oxidant and anti-inflammatory properties.^{4,5}

Licorice is a common herb which has been widely used for centuries in traditional Chinese medicine and a large variety of triterpenoids and flavonoids with pharmacological activities have been extracted from its roots or rhizomes. Liquiritigenin (LTG) is one of the most interesting active flavonoid molecule extracted from licorice roots (*Glycyrrhiza glabra*, Fabacea).⁶ LTG has been reported to have hepato-protective, neuro-protective, anti-inflammatory, anti-tumoral *in vitro*⁷ even if *in vivo* studies suggest that LTG acts only as selective agonist for the beta subtype of the estrogen receptor (ER-β).^{8,9} There are many studies in which the effects of LTG on bone cells have been investigated, identifying positive effects on osteoblastic mineralization activity, collagen synthesis, alkaline phosphatase (ALP) activity¹⁰ and protection from the oxidative stress,¹¹ as well as inhibition of the osteoclastic differentiation.¹²

* Corresponding author. IRCCS Orthopedic Institute Galeazzi, Via R. Galeazzi 4, 20161, Milan, Italy.

E-mail address: massimo.mariotti@unimi.it (M. Mariotti).

Peer review under responsibility of Japanese Pharmacological Society.

Danio rerio (zebrafish) is a powerful model for translational studies on human bone diseases. In particular, zebrafish embryo is a widely used model to study developmental osteogenesis¹³ while zebrafish scale represents a very helpful model due to its accessibility, easy handling, quantity and similarity to human bone. Indeed, the scale is constituted by calcium phosphate and hydroxyapatite crystals forming a structure very similar to human woven bone¹⁴ with the same cell type and regulatory mechanisms of deposition and resorption of human tissue.^{15,16} The glucocorticoid prednisolone (PN) induces a dose-dependent loss of mineral matrix generating glucocorticoid-induced osteoporosis (GIOP) that can be evaluated in zebrafish scales through histological and biochemical tests. Zebrafish GIOP scales are characterized by reduced mineral content and by the presence of resorption lacunae very similar to those observed in the osteoporotic human bones. PN treatment induces alterations in bone markers such as increased tartrate resistant acid phosphatase (TRAP) activity and decreased ALP activity that can be easily evaluated in zebrafish scales.¹⁷ Zebrafish GIOP model can be used to test the effectiveness of natural molecules to counteract the onset of PN-dependent effects. The aim of this study was to test the ability of LTG to prevent the formation of the osteoporotic phenotype in scales from zebrafish GIOP model.

2. Material and methods

2.1. Ethic statement

This experimentation has been performed in the Zebrafish Laboratory (IRCCS R. Galeazzi, GSD Foundation, Milan, Italy) according to the Italian and European guidelines on research practice (EU Directive 2010/63/EU). Zebrafish experimentation and all protocols of this study were approved by the Italian Ministry of Health (authorization No.349/2017-PR).

2.2. Chemicals

Liquiritigenin (7,4'-Dihydroxyflavanone, LTG) and prednisolone (1-Dehydrohydrocortisone, PN) have been purchased by Sigma–Aldrich and both were initially dissolved in dimethyl sulfoxide (DMSO, Sigma–Aldrich) then diluted in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄) to have the final concentrations listed below.

2.3. Animals

Adult zebrafish (*D. rerio*) AB strains were maintained in a ZEBTEC® bench top system (Tecniplast) under standard conditions.¹⁸ Embryos have been generated by crossing single mates of adult fish for each single experiment. During the treatments both adult fish and embryos have been maintained at 28 °C in E3 medium.

2.4. Embryo treatments

After the breeding, zebrafish embryos (0 h post fertilization, 0hpf) have been washed for a few minutes with a methylene blue solution (2 ml of 0.1% methylene blue, to 1 L of fish water) to suppresses eventual fungal or mold outbreaks¹⁸ then embryos were checked for general health conditions using a light stereomicroscope (Olympus SZX-ZB7).¹⁹ Embryos have been exposed to a range of LTG concentrations from 1 pM to 10 µM in E3 medium for 6 days; control embryos were maintained in E3 medium and in E3 medium containing the same amount of DMSO used for the highest LTG tested concentration (0,0001%). Ten embryos were treated for

any LTG concentration and were maintained at 28 °C at the dark in incubator (Jouan EB 18 Incubator, 18 L).

2.5. Embryo histochemical analysis

After 6 days of LTG treatment, embryos have been euthanized by hypothermal shock²⁰ then fixed in 3.5% formaldehyde 0.1 M sodium phosphate buffer solution. Embryo were then processed to evaluate osteogenesis using a two-color acid-free staining. The staining has been performed according to Walker and Kimmel method²¹ staining cartilage and bone using Alcian blue 8GX (Sigma Aldrich) and Alizarin Red S (ARS, Sigma Aldrich), respectively. Osteogenesis level has been quantified for every single embryo by counting the number of mineralized vertebral bodies normalized for the body length (N.V./L). Embryos have been analyzed under a light stereomicroscope (Olympus SZX-ZB7) and images have been acquired using a discovery CH30 camera (TiEsseLab).

2.6. Adult treatments and scales collection

Nine months old male zebrafish of comparable length and weight have been treated through direct immersion in E3 medium solutions containing different LTG concentrations, from 10 pM to 100 nM. Each LTG concentration has been tested both alone both in our GIOP adult zebrafish model¹⁷ (80 µM PN). Treatment solutions were changed every 48 h and the whole treatment last 14 days. Control fish were maintained in E3 medium containing the same amount of DMSO used for the highest LTG plus PN tested concentration (0,0011%). At the end of the treatment, fish have been anaesthetized using 0.168 mg/ml tricaine methanesulfonate E3 medium solution¹⁸ to allow scales removal. Scales have been carefully removed from both sides of the fish body operating under a light stereomicroscope (Olympus SZX-ZB7) using Dumont® Stainless steel forceps (Sigma Aldrich).

2.7. Histological TRAP and ALP assays in scales

Histological tartrate resistant acid phosphatase (TRAP) assay has been performed on explanted scales using Leukocytes acid phosphatase (TRAP) detection kit (Sigma Aldrich) following the manufacturer's protocol.

For every different treatment, 5 fish have been tested and were explanted 10 scales for fish. Data are then obtained from 50 scales for each treatment and scales have been histologically stained to count the TRAP positive scales. Histological alkaline phosphatase (ALP) assay has been performed using BCIP®/NBT liquid substrate (Sigma Aldrich) according to manufacturer's protocol.

2.8. Biochemical TRAP and ALP assays in scales

Biochemical TRAP activity was evaluated directly on explanted scales using a test previously published by Perrson et al.¹⁶ while biochemical ALP activity was evaluated according to our method.¹⁵ To perform both these analysis, absorbance has been read at 405 nm using a spectrophotometer (iMark™ Microplate Reader, Bio-Rad).

2.9. Bone matrix vital staining

At the end of the treatment, fish have been live stained using a 0.005% calcein (Bis[*N,N*-bis(carboxymethyl)aminomethyl]fluorescein, Sigma Aldrich) E3 solution.²² Scales have been carefully removed as previously described and fixed using 3.5% formaldehyde 0.1 M sodium phosphate buffer solution. Images were

acquired using a fluorescence microscope (Olympus SZX-ZB7) equipped with Discovery CH30 camera (TiESseLab).

2.10. Statistics

Data concerning embryo histochemical analysis derived from 10 embryos treated for every LTG concentration performing 3 independent experiments which gave comparable results. Data were plotted on SigmaStat3.5 software to be subjected to one-way analysis of variance (ANOVA) followed by Dunn's test for multiple comparisons. Data concerning adult fish experimentation were obtained by the analysis of 10 scales explanted from 5 fish for each tested LTG concentration. Each experiment has been repeated three times with comparable results. The results are expressed as mean of the means of the three independent experiments \pm standard deviation versus control. The statistical significance has been determined for P-values by using ANOVA followed by Bonferroni test for multiple comparisons. All the significance values were set at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

3. Results

3.1. Liguiritigenin treatment enhances osteogenesis in zebrafish embryos

Zebrafish embryos 0hpf were exposed to different LTG concentrations, from 1 pM to 10 μ M, dissolved in E3 medium. After 6 days, embryos have been analyzed to evaluate the mineralization rate by N.V./L. parameter, obtained from the number of the ARS positive vertebral bodies along the embryo length. LTG treatment induced 71% increase of the mineralization rate at 10 nM concentration (Fig. 1A). Fig. 1B shows the effective increase of the number of mineralized vertebral bodies in 10 nM LTG treated embryos versus untreated embryos. Control embryos were maintained in E3 medium containing the same amount of DMSO used for the highest LTG tested concentration (0,0001%) and does not show any

alteration compared to E3 only maintained embryos (data not shown). The general embryo development was not affected by LTG treatment or by DMSO, since body length and cartilage development, stained with Alcian blue, were comparable.

3.2. Bone matrix analysis shows the protective effect of liguiritigenin on PN-induced bone loss in adult fish scales

Adult fish have been treated with different LTG concentrations, from 10 pM to 100 nM, with or without 80 μ M PN and calcein live staining was used to evaluate the integrity of the mineral matrix in the scales. Fig. 2 shows the effects of PN treatment on the scale mineralized matrix since it produces in all scales resorption lacunae along the borders. LTG treatment (1 nM) did not have negative effect on scale mineralized matrix alone whereas it prevented the formation of the resorption lacunae in PN-treated fish where the bone surface of the scale appears intact (Fig. 2).

3.3. Liguiritigenin prevents osteoclast activation in scales of PN-treated fish

The osteoclast resorption activity was evaluated along scale borders by histochemical TRAP assay. As shown in Fig. 3A 100% of scales from PN-treated fish showed intense TRAP activity while untreated fish show TRAP activity only in very few scales (9%). 10 pM and 100 pM LTG concentrations reduced the percentage of TRAP-positive scales (30% and 50% respectively) compared to the 100% positive scales of PN treatment but if given alone, in the absence of PN, they caused a partial osteoclastic activation (40% of TRAP-positive scales) suggesting the presence of some negative effects on scale bone metabolism. 1 nM LTG strongly suppressed PN-dependent osteoclasts activation reducing the percentage of TRAP-positive scales to 10% and if administered alone does not induce osteoclastic activation. Higher LTG concentration show a little TRAP activation in control fish (100 nM) but most importantly does not contrast TRAP activation in PN model since TRAP

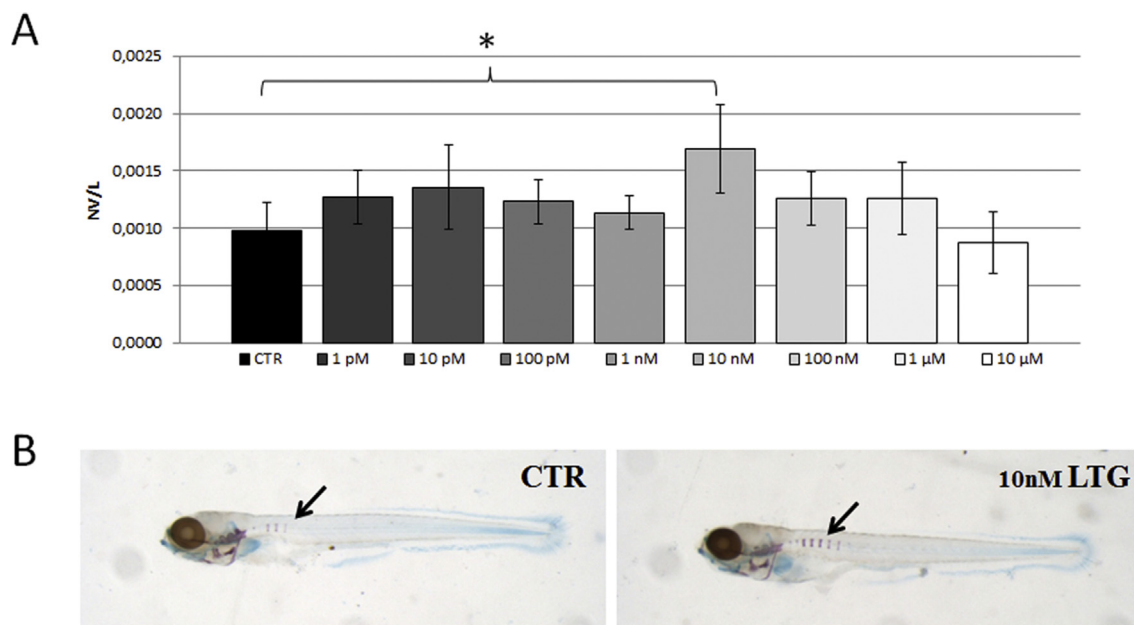


Fig. 1. Mineralization rate in zebrafish embryos treated with different LTG concentrations for 6 days. (A) 10 nM LTG treatment induces an increase of 71% of the mineralization rate respect the untreated embryos (CTR vs. 10 nM LTG, $p < 0.05$). The mineralization rate was evaluated as ratio of the number of mineralized vertebral bodies along body length (NV/L). (B) Alcian blue/ARS acid-free staining highlights an increased number of vertebral bodies (black arrows) in 10 nM LTG embryos vs untreated controls (CTR). No toxicity has been detected. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

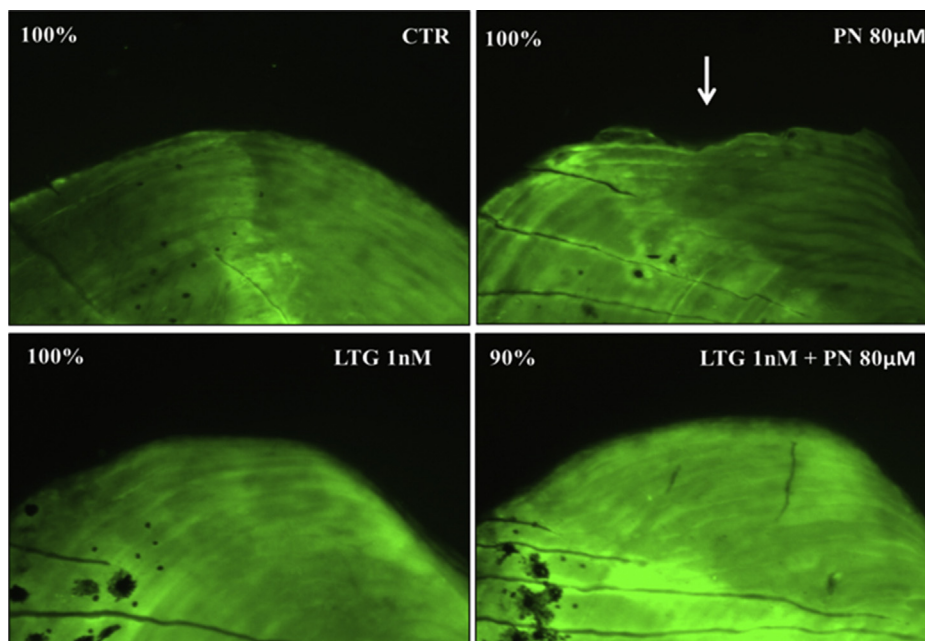


Fig. 2. Calcein vital staining of scales from adult zebrafish treated with LTG \pm PN evaluated for the presence of resorption lacunae (white arrow) along the scale border. The percentage indicates the fraction of the scales with that feature. PN-treated fish show resorption lacunae in all the scales while LTG+PN treated fish show an intact mineralized matrix in 90% of scales.

positive cells are 100%. Histochemical TRAP staining marks the localization and the extension of resorption lacunae which appeared to be much smaller in 1 nM LTG+PN fish compared to PN-treated fish (Fig. 3B). To quantify the resorption activity we performed a TRAP biochemical assay which confirmed that 1 nM LTG treatment prevents the PN-dependent increase of TRAP in zebrafish scales (Fig. 3C).

3.4. Liquiritigenin treatment has no effect on osteoblast activity downregulation in scales of PN-treated fish

Zebrafish GIOP model is also characterized by a decreased osteoblast activity in the scales that can be evaluated through histological and biochemical assays.¹⁷ Biochemical scales analysis confirmed the PN-induced reduction of ALP activity in all the scales whereas 1 nM LTG in addition to PN treatment did not alter this effect (Fig. 4A). Histochemical assay of ALP activity confirmed that the loss of ALP signal in PN-treated scales is not rescued when incubated with 1 nM LTG+PN (Fig. 4B). ALP activity has been evaluated also in fish co-treated with PN and all the others LTG concentrations, from 10pM to 100 nM, showing a persistent low biochemical and histological ALP activity (data not shown).

4. Discussion

Despite the numerous advantages that the zebrafish animal model offers in orthopedic field, very few data have been produced about the effects of natural molecules on fish bones. The innovative read-out system based on fish scale analysis represents a powerful resource to easily monitor the bone metabolism modulation after a pharmacological treatment, like LTG.

LTG is extracted from a variety of plants and especially from licorice roots together with isoliquiritigenin (ISL).⁸ Therefore it must be considered that ISL is a chalcone, the precursor but also the isomer of LTG, which is a flavanone. In aqueous solutions, it has been reported that LTG conversion to ISL, from flavanone to

chalcone and vice versa, can occur so that ISL and LTG are both biosynthetically related, chemically interchangeable and therefore in dynamic equilibrium.⁷ *In vitro* studies reported that both LTG and ISL exhibit hepatoprotective, neuroprotective, anti-inflammatory, anti-tumoral and estrogenic-like activities⁷ whereas *in vivo* studies suggested that the two molecules have different pharmacological properties and effects. In particular, ISL has mainly anti-oxidative, anti-inflammatory, immune-regulatory, anti-tumoral and hepatoprotective activities and it is widely used in many disease prevention and treatment like cancer and antibiotic therapies.⁹ About LTG, it would act only as selective potent agonist for the beta subtype of estrogen receptor (ER- β).^{8,9,23} These data lead to the hypothesis that the protective effects of LTG on zebrafish bone may be therefore due to the estrogen-like activity of LTG itself and/or to the anti-inflammatory and antioxidant activities of its isomer ISL.

A series of *in vitro* and *in vivo* studies highlight that LTG has anabolic activity on bone tissue. LTG promotes osteoblast differentiation *in vitro*, enhances ALP activity¹² and stimulates cell growth and collagen synthesis in murine osteoblastic MC3T3-E1 cells.¹⁰ Taking together, these results support our data concerning the LTG effect on zebrafish embryos mineralization. LTG enhanced the mineralization rate of vertebral bodies in a dose-dependent manner, where 10n M LTG seems to be crucial to obtain a pro-osteogenic effect in zebrafish embryo model. This LTG effect may be exerted directly on mesenchymal stem cell differentiation process, as demonstrated in recent studies.²⁴ Similarly, Licochalcone A, a chalconoid isolated from liquorice root, has shown osteogenic properties enhancing the rate of skeletal development in zebrafish embryo as well as in mouse calvarial bones.²⁵

Glucocorticoids (GCs) administration induces an osteoporotic phenotype in many animal models, such as mouse,²⁶ rat,²⁷ rabbits,²⁸ ewes,²⁹ beagles,³⁰ pigs³¹ and zebrafish.¹⁷

Interestingly, unlike the embryonic osteogenesis, LTG does not modulate ALP activity in adult fish neither in normal scales nor in PN-treated scales. These data suggest that the osteoblast is not a

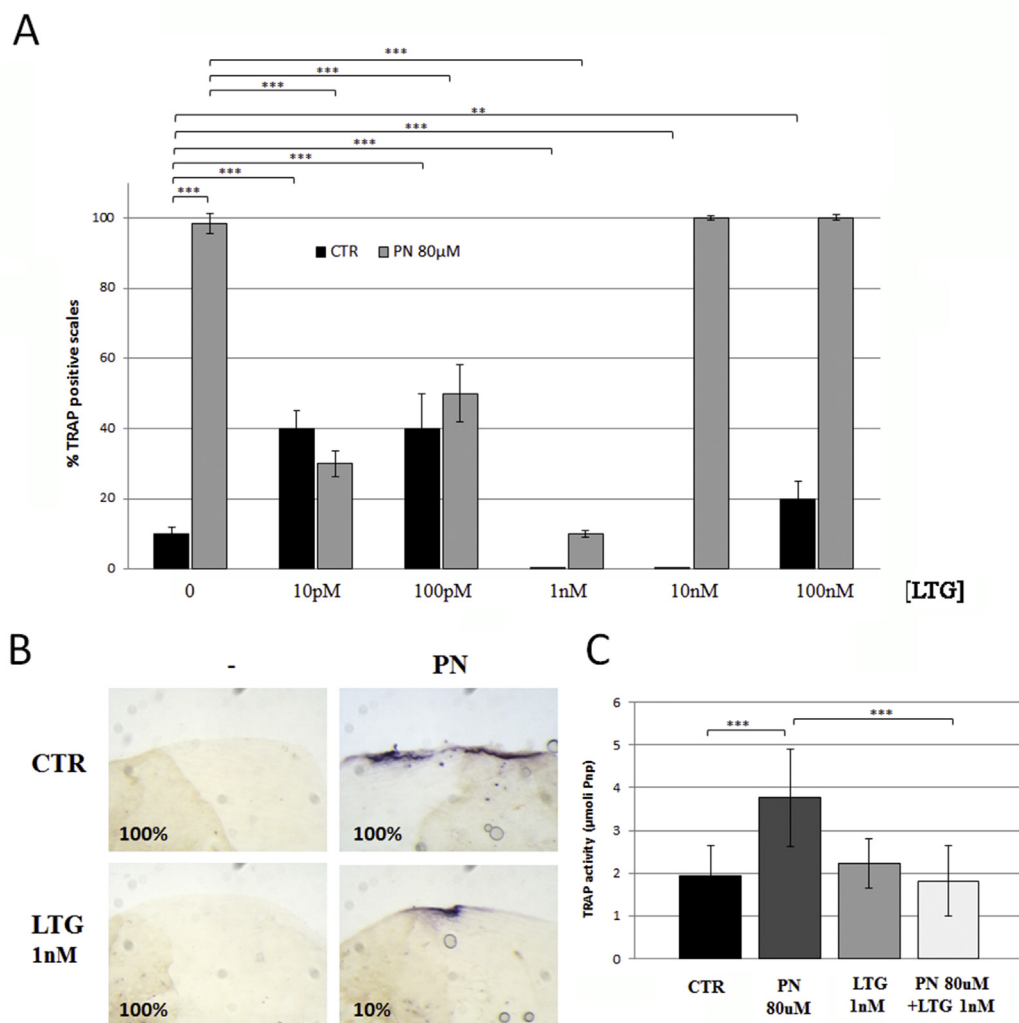


Fig. 3. Analysis of TRAP activity in LTG-treated GIOF fish. **(A)** Percentage of TRAP-positive scales in adult zebrafish treated with different LTG concentrations with (PN vs 10pM LTG, $p < 0.001$; PN vs 100pM LTG, $p < 0.001$; PN vs 1 nM LTG) or without PN (CTR vs 10pM LTG, $p < 0.001$; CTR vs 100pM LTG, $p < 0.001$; CTR vs 1 nM LTG, $p < 0.001$; CTR vs 10 nM LTG, $p < 0.001$; CTR vs 100 nM LRG, $p < 0.01$). PN alone induced TRAP activity in 100% of scales (CTR vs 80 µM PN, $p < 0.001$) whereas 1 nM LTG suppressed PN-dependent TRAP activation (1 nM LTG + PN 80 µM vs PN 80 µM, $p < 0.001$, -90%). **(B)** Histochemical TRAP staining of scale treated with PN shows great resorption lacunae (purple staining) in all scales (100%) while only 10% of scales from 1 nM LTG+PN fish shows slight TRAP activity. **(C)** Biochemical TRAP analysis performed on scales confirms that 1 nM LTG treatment prevents the PN-dependent increase of TRAP activity (PN 80 µM vs CTR, $p < 0.001$, +99%; 1 nM LTG + PN 80 µM vs PN 80 µM, $p < 0.001$, -53%). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

target of LTG *in vivo* during bone remodeling processes in adult stage. It is well known that the embryonic osteogenesis differs from adult bone remodeling by cell types, receptor patterns and humoral signalings. Moreover, mesenchymal stem cells of zebrafish embryo show a very different behavior compared to mature osteoblasts of adult scale and they are able to respond to different stimuli. In fact, many molecules have been demonstrated to show different effects *in vitro* and *in vivo*.

It is known that PN enhances the osteoclasts TRAP activity in zebrafish scales leading to large resorption lacunae on the margin of the scales.¹⁷ LTG treatment at the concentration of 1 nM counteracts the activation of the enzyme TRAP in GIOF model preventing bone-loss in the scale without secondary effects. Also in this case, the dosage seems to be crucial to obtain maximal anti-resorbing effects and low tissue stress. Interestingly, LTG alone at lower concentrations (<1 nM) is slightly pro-osteoporotic and less efficient against PN effects. On contrary, higher concentration (>1 nM) do not show any modulation of TRAP activity in GIOF fish

as well in controls. Calcein live staining of bone matrix confirms that 1 nM LTG treatment prevents the formation of the osteoporotic phenotype protecting the mineralized matrix from resorption.

An *in vitro* study by Rho et al. find out that water extract of *Glycyrrhiza radix* inhibits osteoclast formation in mouse calvariae by downregulation of receptor activator of nuclear factor kappa-B ligand (RANKL) and TRAP mRNA expression as well as other osteoclast specific genes.³² LTG decrease reactive oxygen species (ROS) production and osteoclast differentiation *in vitro* inducing tumor necrosis factor alpha (TNFα), RANKL and interleukin-6.¹⁰ A study by Uchino et al. reports LTG inhibitory effects on osteoclast differentiation of bone-marrow macrophages and activity in MC3T3-E1 cell line with downregulation of important osteoclasts markers proteins such as cathepsin K, proto-oncogene Src and nuclear factor of activated T-cell cytoplasmic-1 (NFATC1).¹² ISL has important effects on osteoclastogenesis and osteoclast activity, too. ISL suppresses RANKL-induced osteoclastogenesis *in vivo* and inhibits *in vitro* the inflammatory bone loss by attenuating the osteoclast activity via

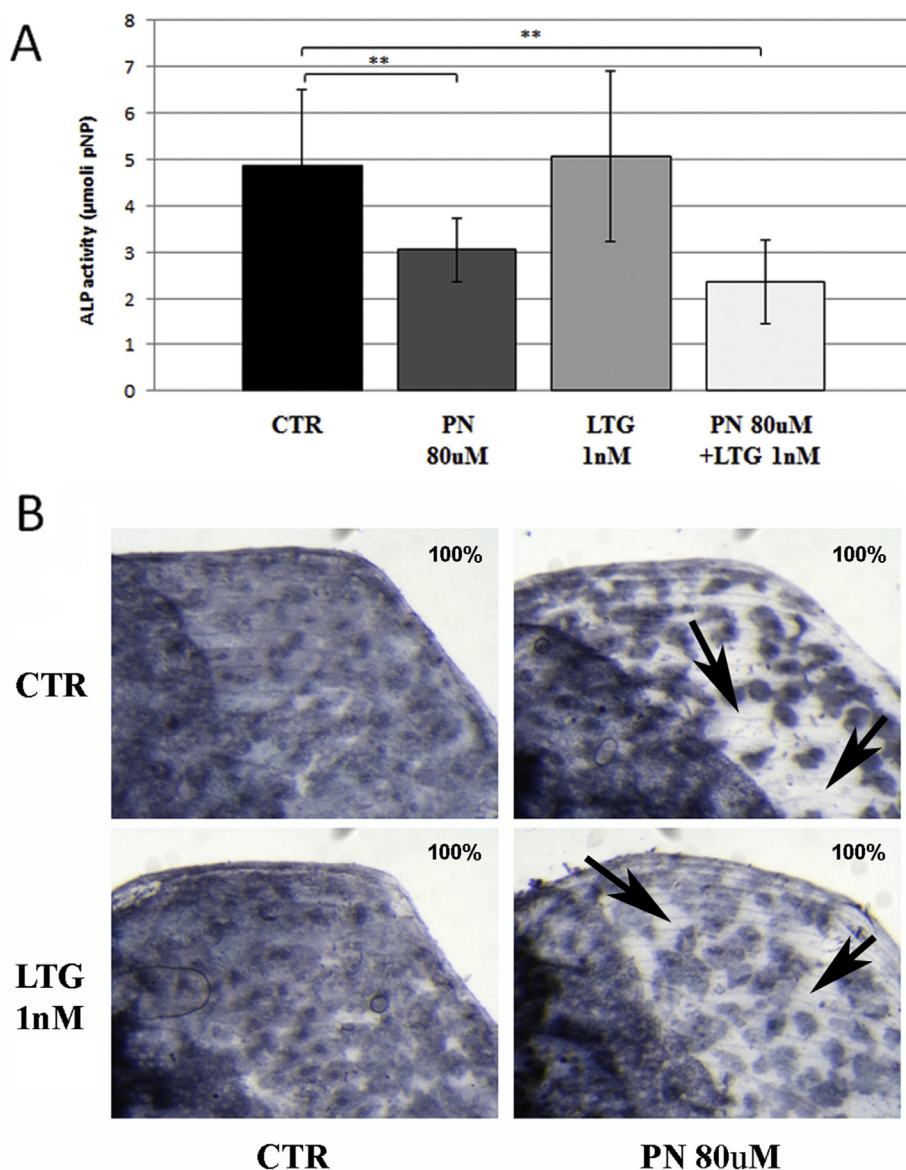


Fig. 4. Biochemical and histochemical ALP assay in scales of fish treated with PN ± LTG. **(A)** In the biochemical ALP assay, PN reduces ALP activity in all the scales whereas co-treatment with 1 nM LTG does not alter this effect (PN 80 μM vs CTR, $p < 0.01$, –38%; 1 nM LTG vs CTR, $p < 0.01$, –52%). **(B)** Histochemical ALP analysis confirms the PN-induced reduction of ALP activity (black arrows) is not modulated by 1 nM LTG. The percentage indicates the fraction of the scales with that feature.

RANKL-RANK-TRAF6, mitogen-activated protein kinases (MAPK), IκBα/NF-κB, and AP-1 signaling pathways.³³ In addition, in *in vitro* studies performed on hFOB1.19 cells stimulated with conditioned medium of MDA-MB-231 cells, ISL significantly reduces the RANKL/osteoprotegerin (OPG) ratio inhibiting RANKL production and restoring normal OPG levels.³⁴

Osteoclasts are therefore strongly influenced by inflammatory factors and it is well established that an inflammatory state and the consequent activation of T-cells are correlated to increased bone resorption. In fact, some cytokines have osteoclastogenic properties and can influence osteoclast behavior directly or indirectly, via the RANK/RANKL/OPG system.³⁵ The anti-inflammatory/antioxidant effects of ISL and LTG on osteoclast activity in fish GIOP model may represent the key function in bone protection.

The amount of LTG seems to be crucial for osteoclast inhibition. In fact, it is not unusual that anti-osteoporotic molecules have effect

only at low dosage. For example, propranolol inhibits osteoclast differentiation at lower dose whereas at higher dosage does not.³⁶

Phytoestrogens are naturally occurring compounds found in plants that mimic human estrogen hormone when taken with diet. These molecules have been suggested to be effective on human bone health against, in particular against post-menopausal osteoporosis.³⁷

However, these compounds may induce or inhibit estrogen-dependent signaling due to their ability in activation/inhibition of the estrogen receptors ER-α or ER-β,³⁸ both expressed in bone cells.

LTG is part of phytoestrogen family and acts as high-affinity agonist of ER-β like other molecules such as genistein, daidzein and equol.³⁹ It has been demonstrated that phytoestrogens with ER-β selectivity can lead to a positive effect on bone cells. For example, genistein is able to induce osteoblast differentiation *in vitro*⁴⁰ and some benefits in bones of ovariectomized rats.⁴¹

Moreover, genistein has been also found to inhibit osteoclast differentiation *in vitro* on RAW246.7 cell line.⁴²

Since ER- β agonists are not the major player in the *in vivo* estrogen protection of adult bone mass,⁴³ LTG, like genistein, may exert the major anti-osteoporotic activity in GIOF fish through anti-inflammatory/antioxidant properties. Nevertheless, a minor role of the phytoestrogen activity cannot be excluded.

5. Conclusions

This is the first evidence of *in vivo* activity of LTG in bone protection. For this reason, LTG represents an excellent candidate to develop a new natural approach against human osteoporosis. Our results will contribute to encourage the *in vivo* studies and the use of molecules of natural origin for the treatment of human bone diseases.

Funding information

This work was supported by 5 × 1000 anno 2017 progetto SoyFish (Grupposandonato Foundation) and Ministry of Health (Ricerca Corrente), Italy.

Authors' contributions

MM designed the study and the experimental procedure and finalized the manuscript for submission. GB received the fund. MC did all the experiments, analyzed the data and prepared the initial version of the manuscript.

Declaration of Competing Interest

The authors have not disclosed any affiliation or financial involvement with organizations or entities with a direct financial interest in the subject matter or materials discussed in the manuscript.

References

- Lin Y, Kazlova V, Ramakrishnan S, et al. Bone health nutraceuticals alter microarray mRNA gene expression: a randomized, parallel, open-label clinical study. *Phytomedicine*. 2016;23:18–26.
- New SA. Intake of fruit and vegetables: implications for bone health. *Proc Nutr Soc*. 2003;62:889–899.
- Tarantino U, Iolascon G, Cianferotti L, et al. Clinical guidelines for the prevention and treatment of osteoporosis: summary statements and recommendations from the Italian Society for Orthopaedics and Traumatology. *J Orthop Traumatol*. 2017;18(Suppl 19):3–36.
- Scorei ID, Scorei RI. Calcium fructoborate helps control inflammation associated with diminished bone health. *Biol Trace Elem Res*. 2013;155:315–321.
- Leong DJ, Choudhury M, Hirsh DM, Hardin JA, Cobelli NJ, Sun HB. Nutraceuticals: potential for chondroprotection and molecular targeting of osteoarthritis. *Int J Mol Sci*. 2013;14:23063–23085.
- Wang L, Yang R, Yuan B, Liu Y, Liu C. The antiviral and antimicrobial activities of licorice, a widely-used Chinese herb. *Acta Pharm Sin B*. 2015;5:310–315.
- Simmler C, Hajirahimkhan A, Lankin DC, et al. Dynamic residual complexity of the isoliquiritigenin–liquiritigenin interconversion during bioassay. *J Agric Food Chem*. 2013;61:2146–2157.
- Gaur R, Yadav KS, Verma RK, Yadav NP, Bhakuni RS. *In vivo* anti-diabetic activity of derivatives of isoliquiritigenin and liquiritigenin. *Phytomedicine*. 2014;21:415–422.
- Peng F, du Q, Peng C, et al. A review: the pharmacology of isoliquiritigenin. *Phytother Res*. 2015;29:969–977.
- Choi EM. Liquiritigenin isolated from *Glycyrrhiza uralensis* stimulates osteoblast function in osteoblastic MC3T3-E1 cells. *Int Immunopharm*. 2012;12:139–143.
- Choi EM, Suh KS, Lee YS. Liquiritigenin restores osteoblast damage through regulating oxidative stress and mitochondrial dysfunction. *Phytother Res*. 2014;28:880–886.
- Uchino K, Okamoto K, Sakai E, et al. Dual effects of liquiritigenin on the proliferation of bone cells: promotion of osteoblast differentiation and inhibition of osteoclast differentiation. *Phytother Res*. 2015;29:1714–1721.
- Kimmel CB, DeLaurier A, Ullmann B, Dowd J, McFadden M. Modes of developmental outgrowth and shaping of a craniofacial bone in zebrafish. *PLoS One*. 2010;5, e9475.
- Sire JY, Akimenko MA. Scale development in fish: a review, with description of sonic hedgehog (shh) expression in the zebrafish (*Danio rerio*). *Int J Dev Biol*. 2004;48:233–247.
- Perrson P, Takagi Y, Björnsson BT. Tartrate resistant acid phosphatases as a marker for scale resorption in rainbow trout, *Oncorhynchus mykiss*: effects of estradiol-17 β treatment and refeeding. *Fish Physiol Biochem*. 1995;14:329–339.
- Pasqualetti S, Banfi G, Mariotti M. Osteoblast and osteoclast behavior in zebrafish cultured scales. *Cell Tissue Res*. 2012;350:69–75.
- Pasqualetti S, Congiu T, Banfi G, Mariotti M. Alendronate rescued osteoporotic phenotype in a model of glucocorticoid-induced osteoporosis in adult zebrafish scale. *Int J Exp Pathol*. 2015;96:11–20.
- Westerfield M. *The zebrafish book, A guide for the laboratory use of zebrafish (Danio rerio)*. 5th ed. Eugene: University of Oregon Press; 2007.
- Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev Dynam*. 1995;203:253–310.
- Matthews M, Varga ZM. Anesthesia and euthanasia in zebrafish. *ILAR J*. 2012;53:192.
- Walker MB, Kimmel CB. A two-color acid-free cartilage and bone stain for zebrafish larvae. *Biotech Histochem*. 2007;82:23–28.
- Carnovali M, Luzzi L, Banfi G, Mariotti M. Chronic hyperglycaemia affects bone metabolism in adult zebrafish scale model. *Endocrine*. 2016;54:808–817.
- Sareddy GR, Vadlamudi RK. Cancer therapy using natural ligands that target estrogen receptor beta. *Chin J Nat Med*. 2015;13:801–807.
- Azizoltani A, Piri K, Behzad S, et al. Ethyl acetate extract of licorice root (*Glycyrrhiza glabra*) enhances proliferation and osteogenic differentiation of human bone marrow mesenchymal stem cells. *Iran J Pharm Res (IJPR)*. 2018;17:1057–1067.
- Kim SN, Bae SJ, Kwak HB, et al. *In vitro* and *in vivo* osteogenic activity of licochalcone A. *Amino Acids*. 2012;42:1455–1465.
- Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC. Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. *J Clin Invest*. 1998;102:274–282.
- Lindgren JU, Johnell O, DeLuca HF. Studies of bone tissue in rats treated by prednisolone and 1,25-(OH) $_2$ D $_3$. *Clin Orthop Relat Res*. 1983;181:264–268.
- Baofeng L, Zhi Y, Bei C, Guolin M, Qinghui Y, Jian L. Characterization of a rabbit osteoporosis model induced by ovariectomy and glucocorticoid. *Acta Orthop*. 2010;81:396–401.
- Chavassieux P, Pastoureaux P, Chapuy MC, Delmas PD, Meunier PJ. Glucocorticoid-induced inhibition of osteoblastic bone formation in ewes: a biochemical and histomorphometric study. *Osteoporos Int*. 1993;3:97–102.
- Quarles LD. Prednisone-induced osteopenia in beagles: variable effects mediated by differential suppression of bone formation. *Am J Physiol*. 1992;263:E136–E141.
- Scholz-ahrens KE, Delling G, Stampa B, et al. Glucocorticosteroid-induced osteoporosis in adult primiparous Gottingen miniature pigs: effects on bone mineral and mineral metabolism. *Am J Physiol Endocrinol Metab*. 2007;293:E385–E395.
- Rho TW, Lee SY, Han SY, et al. Glycyrrhizae radix inhibits osteoclasts differentiation by inhibiting c-Fos-Dependent NFATc1 expression. *Am J Chin Med*. 2017;45:283–298.
- Zhu L, Wei H, Wu Y, et al. Licorice isoliquiritigenin suppresses RANKL-induced osteoclastogenesis *in vitro* and prevents inflammatory bone loss *in vivo*. *Int J Biochem Cell Biol*. 2012;44:1139–1152.
- Ji B, Zhang Z, Guo W, et al. Isoliquiritigenin blunts osteoarthritis by inhibition of bone resorption and angiogenesis in subchondral bone. *Sci Rep*. 2018;8:1721.
- Zupan J, Jeras M, Marc J. Osteoimmunology and the influence of pro-inflammatory cytokines on osteoclasts. *Biochem Med*. 2013;23:43–63.
- Rodrigues WF, Madeira MF, da Silva TA, et al. Low dose of propranolol downmodulates bone resorption by inhibiting inflammation and osteoclast differentiation. *Br J Pharmacol*. 2012;165(7):2140–2151.
- Abdi F, Alimoradi Z, Haqi P, Mahdizad F. Effects of phytoestrogens on bone mineral density during the menopause transition: a systematic review of randomized, controlled trials. *Climacteric*. 2016;19:535–545.
- Mueller SO, Simon S, Chae K, Metzler M, Korach KS. Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor alpha (ERalpha) and ERbeta in human cells. *Toxicol Sci*. 2004;80:14–25.
- Jiang Y, Gong P, Madak-Erdogan Z, et al. Mechanisms enforcing the estrogen receptor β selectivity of botanical estrogens. *Faseb J*. 2013;27:4406–4418.
- Kim M, Lim J, Lee JH, et al. Understanding the functional role of genistein in the bone differentiation in mouse osteoblastic cell line MC3T3-E1 by RNA-seq analysis. *Sci Rep*. 2018;8:3257.
- Reinwald S, Mayer LP, Hoyer PB, Turner CH, Barnes S, Weaver CM. A longitudinal study of the effect of genistein on bone in two different murine models of diminished estrogen-producing capacity. *J Osteoporos*. 2010;2010pii:145170.
- García Palacios V, Robinson LJ, Borysenko CV, Lehmann T, Kalla SE, Blair HC. Negative regulation of RANKL-induced osteoclastic differentiation in RAW264.7 Cells by estrogen and phytoestrogens. *J Biol Chem*. 2005;280:13720–13727.
- Harris HA. Estrogen receptor- β : recent lessons from *in vivo* studies. *Mol Endocrinol*. 2007;21:1–13.