Predicting and Exploring the Mechanisms of Erzhi Pill in Prevention and Treatment of Osteoporosis Based on Network Pharmacology and Zebrafish Experiments

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Background: Erzhi Pill (EZP), a traditional Chinese medicine (TCM) prescription, has been widely applied to improve bone metabolism and treat osteoporosis (OP) in China. However, its effective constituents and mechanisms remain unclear.

Methods: By combining network pharmacology and zebrafish experiments, an integrative method was employed to address this problem. Firstly, the disease targets of OP were collected from two public gene databases. Secondly, the active compounds and drug targets of EZP were obtained from the traditional Chinese medicine systems pharmacology database and analysis platform (TCMSP). Thirdly, a drug-target-disease interaction network was constructed, and the key active components were identified by analyzing the topological characteristics of the network. Finally, these predicted results were tested by zebrafish experiments and compared with those from the literature. Specifically, quercetin as an important representative active component of EZP was applied to wild type and transgenic zebrafish larvae to assess its effects on skull mineralization and osteoplastic differentiation.

Results: Our study identified 72 active compounds, 220 targets and 166 signaling pathways possibly involved in the prevention and treatment of OP by EZP, wherein quercetin, apigenin, daidzein, luteolin, ursoic acid and kaempferol could be the key compounds, while PI3K-Akt signaling pathway, TNF signaling pathway and IL-17 signaling pathway could be the key signaling pathways. The experiments indicated that quercetin attenuated both the decrease of skull mineralization and the inhibition of skull osteoplastic differentiation in zebrafish larvae triggered by dexamethasone.

Conclusion: Our study not only investigated potentially effective constituents and mechanisms of EZP in the prevention and treatment of OP, but also provided a reference for the in-depth research, development and application of TCM.

Keywords: Erzhi Pill, osteoporosis, network pharmacology, mechanisms, zebrafish, quercetin

Introduction
Osteoporosis (OP) is a systemic metabolic bone disease characterized by reduced bone mass and impaired microarchitecture, and has become one of the important public health issues for the elderly and the postmenopausal women.1 Increasing attention has been paid to OP worldwide because it may lead to an increased risk of fractures.2 At present, the prevention and treatment strategies of OP mainly resort to bone resorption inhibitors, bone production enhancers and bone minerals, such as
vitamin D, raloxifene, calcitonin, anabolic, bisphosphonates, and steroids. However, most of these drugs have some shortcomings, such as poor efficacy and serious adverse reactions, and cannot fundamentally improve bone metabolism and restore the dynamic balance of osteogenetic and osteoclastic activities. Therefore, elucidating the molecular mechanisms underlying OP treatment and developing alternative therapies with reduced side effects are essential to obtain more favorable clinical outcomes.

Erzhi Pill (EZP), a Chinese Pharmacopeia-listed herbal preparation composed of Ecliptae Herba (Mohanlian, MHL) and Fructus Ligustri Lucidi (Nvzhenzi, NZZ), has been widely applied in the long-term clinical management of post-menopausal symptoms and osteoporosis. In vivo studies have shown that EZP has anti-OP effect on ovariectomized rat model and glucocorticoid-induced osteoporosis (GIOP) rat model, and shows effects for bone metabolism regulation and alleviation of post-menopausal symptoms in women. However, due to the complexity of the ingredients of traditional Chinese medicine (TCM) prescription and the limits of existing research methods, its effective constituents, the rationality of herbal combination and the pharmacological mechanisms of promoting bone formation, inhibiting bone absorption and improving bone quality have not been fully elucidated.

Network pharmacology is an effective method developed in recent years to comprehensively study the potential active ingredients and action mechanisms of complex drugs. It integrates system biology, multi-direction pharmacology, network analysis and others effectively, and is especially suitable for the study of TCM prescriptions with multi-component, multi-target and multi-mechanism characteristics. Therefore, in this study, network pharmacology was used to explore the effective constituents and possible mechanisms of EZP in preventing and treating OP. Furthermore, zebrafish experiment and literature comparison were conducted in an attempt to confirm the roles of some effective components.

Materials and Methods

Zebrafish Husbandry

Wild type zebrafish larvae (AB strain) and osterix: nlsGFP transgenic zebrafish larvae named tg(sp7: egfp) were provided and raised by the zebrafish platform of clinical research center, Affiliated Hospital of Guangdong Medical University. In tg(sp7: egfp), osteoblasts are specifically marked by green fluorescent protein (GFP) to make osteoblasts visible. The larvae of tg(sp7: egfp) and AB strain were cultured in the egg water (5 mmol/L NaCl, 0.4 mmol/L CaCl2, 0.17 mmol/L KCl, 0.16 mmol/L MgSO4, and 10 ppm methylene blue) and in the egg water containing 30ppm N-phenylthiourea under isothermal conditions at 28.5°C, respectively. All animal procedures were in accordance with the Guangdong Laboratory Animals Monitoring Institute guidelines with the approval of the Animal Research and Ethics Committees of School of Pharmacy, Guangdong Medical University, China (Approval Number:20,171,210).

Drugs, Reagents and Instruments

Dexamethasone (Dex; MP Biomedicals, Santa Ana, CA, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Japan) to obtain stock solution at a concentration of 10mM and stored at −20°C. Quercetin (QU; Tianjinshilang, Tianjin, China; quercetin content 90%) was identified and determined by HPLC with the standard reference (National Institutes for Food and Drug Control, Beijing, China), and dissolved and diluted to a final concentration of 16 μM in egg water before use. Alizarin red (MP Biomedicals, Santa Ana, CA, USA) was used for staining. Small embryo incubator IPP 400 (Memmert, Schwabach, Germany) and Laser confocal microscope TCS SP5II (LSCM; Leica, Hamburg, Germany) were also used in the experiments.

Compounds and Targets of EZP

The chemical components and targets of the two medicinal herbs in EZP were identified from the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, https://tcmspw.com/tcmsp.php). It is one of the most commonly used databases for the study of TCM and provides reliable information about the components and targets of Chinese herbal medicine. Subsequently, Uniprot database (https://www.uniprot.org) was used to convert target names into standard gene symbols.

Disease Target of Osteoporosis

Using “osteoporosis” as the key words, we searched in GeneCards database (https://www.genecards.org/) and Online Mendelian inheritance in man (OMIM, https://omim.org/) database. After that, all the genes obtained from the two databases were normalized, the duplicates were deleted, and the intersection was taken as the target set of OP.
The Drug-Target-Disease Regulatory Network

To illustrate the molecular regulatory mechanism of EZP in the treatment of OP, a visual network of “drug-target-disease” interaction was constructed. The intersection between drug targets of EZP and disease targets of OP were identified as the targets of the network. In addition, bioactive compounds that could target the intersection genes were also selected. By analyzing the topological properties of the primary regulatory network, we identified the key compounds and targets according to degree values of nodes. The visualization of the networks was achieved by use of Cytoscape software.

Functional Enrichment Analysis

Bioinformatic annotation of targets was performed by using the “clusterprofiler” package of R software. The results of Kyoto Encyclopedia of Genes and Genome (KEGG) enrichment analysis were used to reveal the signaling pathways involved in EZP treatment of OP, and the enrichment results with adjusted P value < 0.05 were identified as statistically significant.

Animal Experiment Designs

The 3 day post-fertilization (dpf) zebrafish larvae were randomly divided into various treatment groups (n=12 larvae/2 wells per group) and transferred into 24-well plates containing culture medium. Each well contained 1 mL of medium with compounds of different concentrations. To test whether quercetin can prevent and treat bone damage, from 3 dpf to 9 dpf, the wild zebrafish larvae and tg(sp7: egfp) strain zebrafish larvae were both treated with 10 μM Dex alone or a mixture of 10 μM Dex and different concentrations of quercetin (0.5 μM, 1 μM, 2 μM, 4 μM, 8 μM, 16 μM). Every day, 50% of the medium in each well was replaced with fresh solution. At 9 dpf, larvae were collected and fixed in 4% paraformaldehyde solution. Wild zebrafish larvae were stained with alizarin red to observe the mineralization of skull. Tg(sp7: egfp) strain zebrafish larvae were scanned by LSCM to observe the differentiation of osteoblasts.

Alizarin Red Staining and Quantitative Analysis of Mineralization

At 9 dpf, the AB strain zebrafish larvae were stained with alizarin red as described in previous reports. Briefly, the staining method comprised the steps of fixing in 4% paraformaldehyde for 2 hours, dehydrating in 50% ethanol in PBS for 10 min, and staining with 0.1% alizarin red in 0.5% KOH solution overnight. After being bleached with 1.5% H2O2 in 1% KOH solution for 30 min, the zebrafish larvae were destained with the different mixtures of 0.5% KOH in glycerin (3:1, 1:1, 1:3) in the mentioned order and then preserved in glycerin at 4°C. The method for the quantitation of larval skull mineralization as the same as previously described. Larval head was placed on a slide, and the micrograph of the skull was taken by a M205 FA stereo microscope (Leica, Germany) equipped with a DFC310 FX camera (Leica, Germany). The area and the integral optical density (IOD) of skull alizarin red staining were quantitatively analyzed by Image-Pro Plus image analysis software version 6.0 (IPP 6.0, Media Cybernetics, USA). More than nine pieces of zebrafish were used in each group.

LSCM Scanning and Fluorescence Imaging Quantitative Analysis

At 9 dpf, tg(sp7: egfp) zebrafish larvae were fixed in 4% paraformaldehyde solution for 2 h and preserved in PBS at 4°C. The methods for LSCM scanning and the quantitation of fluorescence were the same as previously described. The larval head was separated and placed in a confocal laser special glass-bottom dish, and then embedded in 1% low-melting point agarose gel. Next, each sample of larval head was scanned for 10 layers with TCS SP5II LSCM (excited at 488 nm, emission from 500 to 550 nm, resolution: 1024 x 1024 pixels square). The high-resolution confocal fluorescence images of all larvae were analyzed using IPP6.0 with the same parameters. More than nine pieces of zebrafish were used in each group.

Statistical Analysis

All the data were presented as the mean ± S.D. The statistical software SPSS 16.0 (IBM Corp, NY, USA) was used to analyze the experimental data. One-way analysis of variance (ANOVA) with Fisher’s PLSD test was used to evaluate the statistical differences among various treatments if the data were normally distributed and had an equivalency of variances. Otherwise, Dunn’s method for post hoc test was used to perform pairwise comparison of treatment groups. If probabilities were less than 0.05 (P<0.05), statistical differences were considered to be significant.
Results

Compounds and Targets of EZP

In total, 162 compounds were obtained from the TCMSP databases. Detailed information on the compounds is presented in Supplementary Table S1. Among these compounds, 119 were obtained from NZZ, 48 from MHL and 5 from both. These compounds acted on 401 targets, of which 220 targets of 72 compounds intersected with the disease targets of OP. Thus, the 220 targets were considered to be the effective compounds of EZP in the treatment of OP. Detailed information on the common targets and their corresponding effective compounds is shown in Supplementary Table S2. The top 15 effective compounds regulating the most number of targets are shown in Table 1.

Drug-Disease Targets Intersection

As many as 4091 disease targets were obtained by searching osteoporosis in the GeneCards and OMIM databases, whose detailed information is listed in Supplementary Table S3. In order to acquire key targets of regulatory network for further mechanism research, the intersection of OP disease targets and EZP drug targets were identified by use of Venn diagram. As shown in Figure 1, 220 targets were identified as both the disease targets and the drug targets.

Construction of Drug-Target-Disease Interaction Network

As shown in Figure 2, a drug-target-disease interaction network was constructed, which included 220 targets, 72 compounds, 2 Chinese herbal medicines and the top 20 KEGG pathways with the greatest importance. The top 15 compounds regulating the most number of disease targets were highlighted, because they may be the key compounds. On the whole, the interaction network visually displayed the regulatory relationship among compounds, targets and biological pathways and provided an opportunity to explore mechanism of EZP in the treatment of OP.

Function Enrichment Results

The results of functional enrichment showed that 220 drug-disease targets were significantly enriched in 166 KEGG pathways according to the cutoff value of adjusted P value <0.05, with detailed information in Supplementary Table S4. The 20 most significant KEGG signaling pathways are presented in Figure 3. Among the pathways, PI3K-Akt signaling pathway, TNF signaling pathway, IL-17 signaling pathway, Osteoclast differentiation, MAPK signaling pathway and AGE-RAGE signaling pathway in diabetic complications are closely related to the occurrence and development of OP. The PI3K-Akt signaling pathway regulated by EZP is presented in Figure 4.
Figure 2 The regulatory network of EZP in the treatment of OP. This network contained 72 compounds, 220 targets, 20 significant KEGG pathways and 2 Chinese herbal medicine.

Figure 3 The top 15 most significant KEGG pathways for EZP in the treatment of OP.
Quercetin Attenuates Dex-Induced Decrease of Skull Mineralization in AB Strain Zebrafish Larvae

Bone mineralization is a specific and sensitive indicator of bone formation. Since the zebrafish larvae are transparent, their bones can be observed by alizarin red staining. The bone mineralization can be assessed by analyzing the alizarin red staining area and IOD of skull. As shown in Figure 5 and Table 2, compared to the vehicle control (Veh), there was a significant decrease in the alizarin red staining area and IOD of the skull in AB strain zebrafish larvae treated with Dex at the concentration of 10 μM (P<0.05). Quercetin in dosages ranging from 1μM to 16μM alleviated in a dose-dependent manner the decrease in area and IOD of the skull mineralization in AB strain zebrafish larvae induced by Dex (P<0.05), with the best performance obtained by quercetin at 4 μM.

Osteoblasts in tg(sp7: egfp) zebrafish selectively expressed green fluorescent protein (GFP). The osteoblastic differentiation in tg(sp7: egfp) zebrafish can be reflected by the green fluorescence area and IOD of three-dimensional imaging of zebrafish skull, which was performed by LSCM. As shown in Figure 6 and Table 2, 10 μM Dex also reduced the fluorescence area and IOD (P<0.05). Consistent with the experimental results achieved from AB strain zebrafish larvae, quercetin at concentrations from 1 to 16 μM attenuated Dex-induced reduction of the green fluorescence area and IOD in tg(sp7: egfp) zebrafish larvae (P<0.05), with the best performance obtained by quercetin at 4 μM.

Discussion

Results of this study suggested that 7 compounds in EZP acted on at least 36 OP-related targets, so it was speculated that these could be the main effective compounds of EZP in
the treatment of OP. Previous studies have shown that apigenin can promote the formation of osteoblasts and inhibit the differentiation of osteoclasts. Daidzein stimulates osteogenesis through facilitating proliferation, differentiation, and antiapoptosis in human osteoblast-like MG-63 cells via activation of MEK/ERK and PI3K/Akt pathways in an ER-dependent manner. Luteolin promotes osteoblast differentiation by regulating the ERK/Lrp-5/GSK-3β pathway and prevents bone loss in postmenopausal osteoporosis by inhibiting osteoclast differentiation and function.

**Table 2** Protective Effects of QU Against Dex-Induced Inhibition of Skull Mineralization and Osteoblast Differentiation in AB Strain and Tg(sp7: egfp) Larval Zebrafish, Respectively (n ≥9)

| Group          | Mineralization Area | Mineralization IOD | Fluorescence Area | Fluorescence IOD |
|----------------|---------------------|--------------------|-------------------|-------------------|
| Veh            | 10,163±1562         | 2635±408           | 9883±1052         | 2926±259          |
| Dex            | 6195±908*           | 1517±262*          | 6120±1164*        | 1731±395*         |
| D+QU0.5        | 6453±1163           | 1582±266           | 6084±1098         | 1695±231          |
| D+QU1          | 7071±1196           | 1760±226*          | 6914±726          | 2029±239*         |
| D+ QU2         | 7513±1053"          | 1832±184"          | 8392±1640"        | 2543±538"         |
| D+ QU4         | 8421±1226"          | 1956±407"          | 8956±1406"        | 2686±436"         |
| D+ QU8         | 7684±1108"          | 1886±289"          | 8206±1182"        | 2507±478"         |
| D+ QU16        | 7241±766"           | 1711±214"          | 7653±1056"        | 2147±318"         |

Notes: Veh (vehicle control, 0.1% dimethyl sulfoxide); Dex (Dex 10μM); D+QU0.5 (Dex+ QU 0.5μM); D+QU1 (Dex+QU 1μM); D+QU2 (Dex+QU 2μM); D+QU4 (Dex +QU 4μM); D+QU8 (Dex+QU 8μM); D+QU16 (Dex+QU 16μM). Data are given as mean±SD. *P<0.05 vs vehicle control. **P<0.05 vs Dex treatment.

Abbreviations: EZP, Erzhi Pill; OP, osteoporosis; TCMSP, Traditional Chinese Medicine Systems Pharmacology Database; TCM, traditional Chinese medicine; QU, quercetin; OMIM, Online Mendelian inheritance in man; KEGG, Kyoto Encyclopedia of Genes and Genome; Dex, dexamethasone; IOD, integral optical density.
Ursolic acid can promote bone formation, improve the activity of osteoblasts and inhibit the activity of osteoclasts so as to prevent OP.\textsuperscript{29,30} Kaempferol stimulates bone marrow mesenchymal stem cells (BMSCs) to differentiate into osteoblasts by regulating mTOR pathway,\textsuperscript{31} and stimulates estrogen signaling followed by WNT pathway activation to achieve its potential for bone-sparing effects.\textsuperscript{32} Notably, nicotine, the active compound predicted in this study, has a variety of pharmacological activities and is thought to contribute to the onset of several diseases, including OP.\textsuperscript{33} However, several studies suggest that nicotine has a bimodal effect on the proliferation and osteoblast differentiation in human alveolar bone marrow-derived mesenchymal stem cells (hABMMSCs).\textsuperscript{34} Moreover, low-level nicotine has preventive efforts on OP by stimulating osteoblasts proliferation and differentiation.\textsuperscript{35} In summary, the predicted results of this study are in good agreement with and reasonably supported by those reported in the literature.

As the compound with the most targets predicted in this study, quercetin has multiple pharmacological effects, including antioxidant, free radical scavenging, anti-tumor, anti-inflammatory, antibacterial and antiviral activities.\textsuperscript{36} Related studies have shown that quercetin can promote the proliferation and osteogenic differentiation of BMSCs in vitro,\textsuperscript{37} and has a good therapeutic effect on diabetic or postmenopausal osteoporosis rats.\textsuperscript{38,39} However, there are few studies on the effects of quercetin on bone mineralization and osteoblast differentiation in zebrafish. As for the other active compounds of EZP obtained in this study, we will study their roles in the prevention of osteoporosis in the next experiment.

The active ingredients of TCM are the important source of anti-osteoporosis drugs. At present, it is still difficult to screen the anti-osteoporotic components from natural drugs quickly and effectively, due to the deficiency of the current drug screening models. For example, the cell model does not reflect the relationship between cells and...
tissues, so it is difficult to reflect the overall effect of drug’s multi-target action, while the mammalian model has a long experimental period and low sensitivity, and it is difficult to study the mechanism of active components. Zebrafish is a new model organism for bone research. Studies have shown that not only the genomes between zebrafish and human being are highly homologous, but also the molecular mechanisms in the process of bone growth and development between them are very similar. Zebrafish model has the characteristics of both in vivo and in vitro models, and is suitable for both high-throughput screening and pharmacological mechanism research on active ingredients at the overall animal level. Therefore, as an important supplement to cell model and mammalian model, zebrafish model has played an increasingly important role in drug research. Compared with mammalian models, zebrafish has the advantages of small size, strong reproductive ability, rapid bone development, and transparent body of juveniles, so it is easy to observe zebrafish’s bone development. In recent years, zebrafish has been widely used in the study of bone development and screening of bone-protecting drugs. Tg(sp7: egfp) is a hard-bone transgenic zebrafish strain that utilizes the gene promoter of osterix to drive the enhanced GFP expression in osteoblasts at high intensity. Osteoblasts with positive expression of GFP are visible in transparent zebrafish larvae. Therefore, direct monitoring of changes in green fluorescence in tg(sp7: egfp) bone by fluorescence microscope can reflect the differentiation of osteoblasts. In this study, the effects of quercetin combined with Dex on the skull mineralization and expression of GFP in zebrafish larvae were investigated. The results showed that quercetin could promote the differentiation and mineralization of osteoblasts in a concentration-dependent manner within a certain concentration range, thus reducing the bone damage caused by Dex. Related studies have shown that the pharmacological effects of quercetin are concentration-dependent. It induces mesenchymal stem cells to differentiate into osteoblasts at high concentrations and adipocytes at low concentrations. Admittedly, more studies are needed to elucidate the concentration-dependent pharmacological mechanism of QU in the treatment of OP.

Although we applied network pharmacology and zebrafish model experiments to predict and partially verify the effective ingredients and mechanisms of EZP on anti-osteoporosis in this study, there are some limitations. For example, most of the predicted active components and related signaling pathways were not verified in this study. In the future, we will verify more predicted components as well as optimize the compatibility of different components, so as to clarify the effective components of EZP on preventing and treating osteoporosis. On this basis, through cell and rat experiments to explore the role of PI3K-AKT pathway and other related pathways, we will further verify the anti-osteoporosis mechanisms of EZP.

Conclusions
We used the network pharmacology method to screen out the active ingredients and related targets of EZP. By establishing a drug-target-disease interaction network, the effective components and mechanisms of EZP for the treatment of OP were preliminarily explored. The prediction results were partially confirmed by zebrafish experiments and literature studies. Our research showed that EZP in the treatment of OP could involve 72 active compounds, 220 targets and 166 signaling pathways, wherein quercetin, apigenin, daidzein, luteolin, ursolic acid and kaempferol could be the key compounds, while PI3K-Akt signaling pathway, TNF signaling pathway and IL-17 signaling pathway may be the key signaling pathways. Zebrafish experiments showed that quercetin attenuated the decrease of skull mineralization and the inhibition of osteoblastic differentiation in zebrafish larvae triggered by Dex. These findings fully reflected the multi-component, multi-target and multi-mechanism characteristics of TCM in the treatment of diseases. In conclusion, this study not only provided a new insight into the effective constituents and pharmacological effects of EZP in the prevention and treatment of OP, but also provided a reference for the in-depth research, development and application of the traditional medicines.

Data Sharing Statement
The data that support the findings of the present study are available in Supplementary Materials.

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Author Contributions
All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or
critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work. Zhiguo Zhong and Yuyun Li are the co-first authors.

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Disclosure

All authors declare that they have no conflicts of interest for this work.

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