Based on Network Pharmacology to Explore the Mechanisms of *Radix Astragali* Combined with *Prepared Radix Rehmanniain* for Treating Osteoporosis

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Abstract

Background: With the improvement of people's living standards, the aging population in China has gradually increased, and the treatment of osteoporosis (OP) has become a major problem afflicting the medical field. *Radix Astragali*(RA) and *Prepared Radix Rehmannia*(PRR) are commonly used Chinese herbal medicines. The combination of them can achieve a synergistic effect in the treatment of osteoporosis. However, its mechanism of action remains uncertain.

Objective: This study aims to investigate the possible molecular mechanism of RA combined with PRR in the treatment of OP using an integrated strategy of network pharmacology and experimental validation.

Methods: The active ingredients of RA combined with PRR were searched and screened by TCMSP database, and the targets of active ingredients were predicted and supplemented by TCMSP and SwissTargetPrediction databases. The target genes related to OP diseases were searched in GeneCards and OMIM comprehensive databases. The intersection targets of drugs and diseases were imported into String database to obtain the interaction information of intersection target genes. Cytoscape3.7.1 software was used to construct the protein interaction network diagram and "drug component-target-disease" network diagram. Then DAVID database was used for GO gene enrichment analysis and KEGG metabolic pathway analysis. Finally, in vivo experiments were also performed to validate the findings of network pharmacology.

Results: A total of 98 active components of RA combined with PRR were finally retrieved and integrated into the TCMSP database, 1700 target genes related to OP were obtained through the disease database, 149 gene targets were obtained by taking the intersection of disease genes, and drug targets, 122 core targets and 514 interaction relationships were obtained after protein interaction network and topology analysis. GO analysis and KEGG pathway enrichment analysis showed that RA combined with PRR intervention for OP mainly through multiple pathways such as PI3K-Akt, MAPK, TNF, Rap1, and Toll-like receptors. In addition, in vivo experiments confirmed that RA combined with PRR could significantly increase bone mineral density, reduce bone spacing, improve bone tissue structure, and improve osteoporosis in ovariectomized rats.

Conclusion: In this study, the network pharmacological approach was used to reveal the potential targets and key signal pathways of RA combined with PRR in treating osteoporosis. This study was also verified by animal experiments, which provided a reliable basis for clinical application.

Introduction

Osteoporosis (OP) is a metabolic bone disease known as "silent disease." It is mainly a systemic metabolic disease prone to fracture due to the loss and reduction of bone mass, the destruction of bone microstructure, and the increase of bone fragility[1]. With the aging of the world population, the incidence of OP has been on the rise. OP has brought a huge negative impact on patients' health, leading to a sharp decline in the quality of life of middle-aged and older adults. OP is mainly due to the reduction of

estrogen levels after menopause, resulting in insufficient estrogen secretion in the body, so the probability of women suffering from this disease in China is higher than that in men[2]. Its incidence is closely related to age and gender, and reports have shown that the probability of osteoporosis in the elderly over 60 years of age in China is 36%, including 23% in males and 49% in females, which indicates that osteoporosis has become an important public health problem[3]. At present, for patients with osteoporosis, western medicine treatment is mainly based on diet and exercise combined with bone health supplements, bone resorption, and osteoclast inhibitors, and sex hormone supplements at the same time[4]. However, these drugs have significant limitations in treating osteoporosis, such as high price, significant side effects, and low tolerance, so it is difficult to achieve the expected curative effect of patients. Traditional Chinese medicine has a long history of treating osteoporosis. It has the advantages of minor toxic and side effects, moderate price, syndrome differentiation, and overall treatment according to the patient's condition and specific treatment[5]. The theory of traditional Chinese medicine believes that the etiology and pathogenesis of osteoporosis are related to the deficiency of the spleen and kidney[6]. The treatment of osteoporosis is mainly based on the main principles of tonifying the kidney and strengthening the bone, invigorating the spleen, and nourishing the blood. In the process of traditional medical treatment, the compatibility of drug pairs is often carried out to achieve the purpose of adapting to complex conditions, enhancing the efficacy of drugs, and reducing the toxic and side effects of drugs. RA combined with PRR significantly improves osteoporosis, which is better than using a single medicine[7].

RA is a traditional Chinese medicine that was first recorded in "Sheng Nong's herbal classic." It is the dried root of *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao or *Astragalus* membranaceus (Fisch.) Bge. It has the effects of invigorating the spleen to benefit the lung, generating fluid and nourishing blood, astringeting sores, and generating muscles. It is often used to treat clinical diseases such as spleen and lung qi deficiency and blood deficiency. Modern clinical pharmacological studies show that RA has the functions of protecting the kidney, anti-aging, anti-inflammatory, enhancing immunity, preventing and treating osteoporosis, antioxidant stress, and so on[8]. PRR is a processed product of *Rehmannia glutinosa* Libosch. It has the effects of nourishing blood and Yin, benefiting essence and filling marrow. Clinically, it is often used to treat liver and kidney yin deficiency, blood deficiency, waist and knee weakness, etc. According to the results of modern pharmacology and clinical research, PPR has the effects of preventing and treating osteoporosis, regulating immunity, anti-aging, and anti-anxiety [9].

Traditional Chinese medicine has the characteristics of multiple components, multiple targets, and multiple pathways, which makes it difficult to clarify the material basis and mechanism of action of traditional Chinese medicine. The lack of a scientific evaluation system greatly limits the development of Chinese medicine. The holistic and systematic characteristics of network pharmacology are similar to the holistic view of traditional Chinese medicine and the principles of syndrome differentiation and treatment. It embodies the comprehensive effect of traditional Chinese medicine with multiple targets, multiple components, and multiple pathways. It has been widely used to reveal the mechanism of action of traditional Chinese medicine. In this paper, network pharmacology research methods were used to

construct a multi-level network diagram to explore the potential mechanism of RA combined with PRR in treating osteoporosis and further provide a new method and reference for clinical treatment of osteoporosis. The system flow chart is shown in Fig. 1.

Materials And Methods

Screening of Active Ingredients and Targets

The Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, http://tcmspw.com/tcmsp.php) contains 499 herbal medicines and more than 29,000 compound components of each herbal medicine. In this study, the effective ingredients of the "RA combined with PRR" drug pair were retrieved and collected through the pharmacological system database and traditional Chinese medicine analysis platform. The active ingredients that cannot be retrieved in the TCMSP database were supplemented by the SwissTargetPrediction database (http://www.swisstargetprediction.ch/) with "human" as the research species. Finally, all active ingredients were checked and integrated with RA and PRR components.

Acquisition of osteoporosis targets

GeneCards database (https://www.genecards.org/) is a database that includes the human genome, transcriptome, and proteome. The OMIM database (http://www.omim.org/search/advanced/geneMap) is a database on human genes and genetic disorders. In the GeneCards and OMIM comprehensive databases, "osteoporosis" was used as the search term to search the target genes related to osteoporosis diseases, remove duplicate genes, integrate all targets, and establish an osteoporosis disease gene database.

Drawing of Venn diagram

The target protein names searched in the TCMSP database were converted to gene ID names using the UniProt database (https://www.uniprot.org/), and the race was set to "Human." Drew a Venn diagram after obtaining the key targets for the treatment of osteoporosis.

Construction of "active ingredient-target" network

The targets of the intersection of drugs and osteoporosis were imported into the String database (https://string-db.org/) to obtain the interactive information of the target genes, and the species was set to "Homosapiens." Then the active ingredients were imported into Cytoscape 3.7.1 software to construct a "drug ingredient-target" network diagram.

GO gene analysis and KEGG pathway enrichment analysis

DAVID database (https://david.ncifcrf.gov/) is a bioinformatics database, which most commonly performs gene function enrichment analysis. This study used the DAVID database to input the key targets of drugs and diseases, followed by Go gene enrichment analysis and KEGG metabolic pathway analysis.

After integrating and ranking the genes, they were visualized and processed with the ImaGP online mapping website.

Experimental Validation

Experimental animals

Sixty-six healthy SPF SD female rats were purchased from Chengdu Dashuo Laboratory Animal Co., Ltd., with a bodyweight of about 180 ~ 200g and certificate number of SCXK (Chuan) 2020-030. The rats were adaptively fed for seven days in the animal room of the School of Pharmacy, Shaanxi University of Traditional Chinese Medicine before the experiment, randomly divided into six cages, with free diet and water, room temperature of about 25℃, the humidity of about 50%, and kept ventilated in the room. The animal experiment was performed by the guidelines for animal experiments of Shaanxi University of Traditional Chinese Medicine.

Drugs and Reagents

RA (batch number:200801A02, manufacturer: Guangdong Chengchun Pharmaceutical Co, Ltd.), PRR (batch number:200630, manufacturer: Jianzhou Yonggang Herbal Pieces Factory Co., Ltd.), alendronate sodium (batch number: 007200402, manufacturer: Shiyao Group Ouyi Pharmaceutical Co., Ltd., strength:10 mg×6 tablets) were purchased from Xianyang People's Pharmacy of Shaanxi Province; chloral hydrate (manufacturer: Shanghai Shanpu Chemical Co., Ltd.); penicillin injection (batch number: 200702, manufacturer: North China Pharmaceutical Group Animal Health Products Co, Ltd.

Preparation of Experimental Drugs

The decoctions of RA and PRR were prepared by the water decocting method and added 10 times of water to 200g RA and PRR to decoct for 50min and then filtered. Added 8 times of water to decoct for 40 minutes and combined the obtained filtrate (2g/ml crude drug). Since the positive drug alendronate is a tablet, 6 tablets need to be ground into a powder with a mortar, then dissolved in 666ml of pure water, and refrigerated at 4°C for later use.

Grouping and administration of experimental animals

Sixty-six SD female rats were randomly divided into 6 groups of 11 rats each. They were: RA group, PRR group, RA combined with PRR group, positive control group, model group, and sham operation group. On the 7th day after the completion of model establishment, intragastric administration was started. RA group, PRR group and RA combined with PRR group were infused with RA aqueous decoction and PRR aqueous decoction (5.4g·kg^{-1}), RA combined with PRR aqueous decoction (RA 2.7g·kg^{-1} and PRR 2.7g·kg^{-1}, respectively), positive control group (alendronate 1.05mg·kg^{-1}). The model group and sham operation group were intragastrically administered with an equal volume of pure water.

Preparation of Osteoporosis Model Rats

Ovariectomy castration modeling (back ovariectomy) was performed in all rats except the blank group. The rats were fasted but could not help but water 12 hours before modeling. After intraperitoneal injection of chloral hydrate anesthesia, the dorsal skin was performed. A longitudinal incision was made about 0.5-1.0cm beside the midline of the back to find the ovaries and remove them. Penicillin was administered intramuscularly for 3 days postoperatively. The rats were given intragastric administration on the 7th day after the model was established, and the vaginal exfoliated cells were smeared and observed under a microscope on the 10th day.

Detection of Serum Markers

After blood sampling from the abdominal aorta, serum was obtained by centrifugation for serum bone metabolism markers β-CTX, BGP, MSTN, and PIIINP. The detection methods were determined according to the instructions of the ELISA kit. Use an automatic microplate reader to detect the absorbance at 450 nm, record the value, and then convert the content of the marker according to the standard curve.

Bone Mineral Density (BMD) Analysis

After 12 weeks of administration, animals in each group were weighed. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital. After the scanning conditions were set, the lumbar vertebra and left tibia parameters were analyzed.

Pathological Changes in Rat Bone Tissue

The left femur of the rats was fixed with 10% formalin and decalcified with 10% EDTA. The specimens were embedded and cut into slices with a thickness of 4 μm. After staining with hematoxylin-eosin staining solution, it was observed under a microscope.

Results

Screening results of active ingredients

In the TCMSP database, "RA or Huangqi, PRR or Shudi" was used as the search terms, respectively. The search conditions were: oral bioavailability (OB)≥30%, drug-like (DL)≥0.18. Finally, 21 main chemical components of RA and 77 chemical components of PRR were selected. The active ingredients that could not be retrieved in the TCMSP database supplemented their target information through the SwissTargetPrediction database. A total of 98 active ingredients were obtained by focusing and integrating the retrieved RA with all active ingredients of PRR, and the results are shown in Table 1 and Table S1.

Table 1
Basic information of active ingredients in RA combined with PRR

Herb	MOL ID	Molecule Name	OB	DL
	MOL003732	rehmaglutin d	57.03	0.1
PRR	MOL003713	jioglutoside b-qt	89.22	0.08
	MOL003731	rehmaglutin a	29.7	0.1
	MOL003735	aucubin	4.17	0.33
	MOL003720	purpureaside c	3.14	0.38
	MOL003707	jioglutin c	2.55	0.13
	MOL003711	jioglutoside a	3.92	0.39
	MOL000748	hmf	45.07	0.02
	MOL003714	jionoside a	3.62	0.36
	MOL003715	jionoside b	4.27	0.35
RA	MOL000438	(3r)-3-(2-hydroxy-3,4-dimethoxyphenyl)chroman-7-ol	67.67	0.26
	MOL000098	quercetin	46.43	0.28
	MOL000398	isoflavanone	109.99	0.3
	MOL000374	5'-hydroxyiso-muronulatol-2',5'-di-o-glucoside	41.72	0.69
	MOL000387	bifendate	31.1	0.67
	MOL000354	isorhamnetin	49.6	0.31
	MOL000442	1,7-dihydroxy-3,9-dimethoxy pterocarpene	39.05	0.48
	MOL000296	hederagenin	36.91	0.75
	MOL000371	3,9-di-o-methylnissolin	53.74	0.48
	MOL000392	formononetin	69.67	0.21

Screening results of targets

By searching the disease target genes in GeneCards, OMIM databases, 1108 and 592 disease targets of osteoporosis were obtained, respectively, integrated with ranking. A total of 149 intersections of RA combined with PRR drugs on genes and osteoporosis disease target genes were taken. The gene intersection was input in Microbial Information software to draw Venn diagrams of drug and disease targets, as shown in Fig. 2A.

Construction of the “active ingredient-core target” network

The selected active ingredients of the drug and related targets of action were imported into Cytoscape3.7.1 software to construct a "drug-active component-target of action" network diagram. Among them, different colors are used to indicate that they come from different components. The green part in the figure is the target of RA combined with PRR, the purple part represents the active ingredients of RA, and the orange part represents the active ingredients of PRR. The area of the node indicates that the larger the degree value and the more critical the biological function of the node, as shown in Fig. 2B.

Protein interaction network (PPI) analysis

The 149 intersection targets of the interaction between the target genes of active components of RA and PRR and the target genes of osteoporosis were input into string software for protein interaction analysis. Then, the downloaded TSV file was imported into Cytoscape 3.7.1 software, and the "network analysis" function was used for network topology analysis. A total of 122 nodes and 514 edges were obtained. The average betweenness centrality, closeness centrality, and degree values are 0.0161, 0.3562, and 8.4262, respectively. With the help of this software, the drug active ingredient disease target network and PPI network diagram were analyzed to construct the intersection target PPI network diagram (Fig. 2C). The top 10 of the degree value is used as the core target of the network using the CytoHubba plug-in, as shown in Fig. 2D.

GO gene analysis and KEGG pathway enrichment analysis

The genes were integrated and ranked, and a total of 812 entries with $p<0.05$ were obtained after GO gene analysis and screening, including 644 biological process (BP) enrichment results, 47 cell composition (CC) enrichment results, and 121 molecular function (MF) enrichment results. The top 20 BP, CC, and MF genes were used to draw the bubble diagram (Fig. 3A-C). Through KEGG pathway enrichment analysis, a total of 114 signaling pathways with $p<0.05$ were obtained after screening. The results of enrichment analysis showed that RA combined with PRR mainly involved multiple signaling pathways such as PI3K-Akt, MAPK, TNF, Rap1, and Toll-like receptor in the treatment of osteoporosis, are shown in Fig. 3D.

Changes of blood biochemical indicators

Compared with the sham operation group, the BGP level of the model group was significantly reduced, while β-CTX, MSTN, and PIIINP were significantly increased ($P<0.01$). Compared with the model group, the BGP level of the RA and PRR groups was significantly increased ($P<0.05$), the levels of β-CTX, MSTN, and PIIINP were significantly reduced ($P<0.05$). It showed that RA combined with PRR could improve the abnormality of bone formation markers of osteoporosis (Tab 2 and Fig. 4).

Table 2
Effect of RA combined with PRR on BGP、β-CTX、MSTN、PIIINP in serum

Grouping	BGP(ng/ml)	β-CTX(ng/ml)	MSTN(ng/ml)	PIIINP(ng/ml)
SHAM	6.65±2.04	14.06±2.62	5.13±1.04	26.36±4.68
MODEL	3.51±1.03$^{\#\#}$	35.87±6.86$^{\#\#}$	7.92±2.07$^{\#\#}$	40.77±4.03$^{\#\#}$
AS	5.79±1.08**	21.23±3.95**	5.64±1.01*	35.37±4.82*
RA+PRR	6.25±1.24**	23.19±3.76**	5.44±0.73**	28.02±5.77**
PRR	5.45±0.95**	28.73±5.42*	6.0±1.2*	30.07±3.87**
RA	5.03±1.38*	26.86±2.13**	5.91±0.80*	30.86±2.78**

Note: vs the sham operated group, $^{\#}P$<0.05, $^{\#\#}P$<0.01; vs the model group, $^{*}P$<0.05, $^{**}P$<0.01.
Note:vs the sham operated group,#*P*<0.05,##*P*<0.01;vs the model group,**P*<0.05,***P*<0.01.

Pathological changes of bone tissue in rats

The femurs of rats in the sham operation group were mesh-like and neatly arranged, and the bone microstructure was complete. Compared with the sham operation group, the reticular structure of the femur in the model group was destroyed. The bone cortex of the femur of the model group became thin, and the space in the bone marrow cavity increased, indicating that the model group had osteoporosis. Compared with the model group, the number of bones in the RA and PRR groups was significantly increased, the bone tissue structure was significantly improved, and only a few were unevenly arranged (Fig. 5).

Detection of bone mineral density in rats

Compared with the sham operation group, the BMD of the model group's left tibia and lumbar spine was significantly reduced (P<0.01). Compared with the model group, the BMD of rats in the RA, PRR, and RA combined with the PRR group were significantly higher (P<0.05). It was suggested that RA combined with PRR treatment could improve the symptoms of osteoporosis in ovariectomized rats (Tab 3 and Fig. 6).

Table 3
Effect of RA combined with PRR on bone density in ovariectomized rats

Grouping	Lumbar vertebrabone density/g·cm^{-1}	Left tibiabone density/g·cm^{-1}
SHAM	0.162±0.017	0.153±0.026
MODEL	0.142±0.010$^{\#\#}$	0.123±0.015$^{\#\#}$
AS	0.157±0.009**	0.141±0.020*
RA+PRR	0.153±0.011*	0.140±0.018*
PRR	0.152±0.010*	0.136±0.012*
RA	0.148±0.013*	0.138±0.014*

Discussion

Osteoporosis is a systemic bone disease. Due to osteopenia and deterioration of bone microstructure, the risk of fragility fractures increases[10]. The results of modern clinical medical research show that multiple factors cause the pathogenesis of osteoporosis, and the imbalance of bone remodeling is considered to be the main mechanism leading to osteoporosis. Growth hormone activity, musculoskeletal function, daily dietary calcium and vitamin D intake, genetics, and environment can affect bone remodeling and break the bone formation-resorption balance[11]. The myostatin signaling pathway increases bone resorption by activating the RANKL signaling pathway [12].

The results showed that the main active ingredients of RA combined with PRR in treating osteoporosis were mistletoe, kaempferol, isorhamnetin, verbascoside, diosgenin, and catechins. Kaempferol is a flavonol compound with biological effects such as anti-oxidation, anti-tumor, and immune regulation. It can promote the proliferation and differentiation of osteoblasts and increase osteoblast alkaline phosphatase's activity to promote bone formation[13]. Studies have shown that high-dose kanamycin can treat osteoporosis by regulating the balance of Ca2+ metabolism, promoting collagen production, and reducing bone loss[14]. Mistletoe is a kind of polyhydroxyflavonoids, which can effectively promote osteoblast differentiation in MC3T3-E1 cells and inhibit osteoclastogenesis in RAW264.7 cells[15]. At the same time, mistletoe can also promote the proliferation and bone differentiation of bone marrow stem cells[16].

PPI network topology analysis results showed that STAT3, Jun, SRC, AKT1, Mapk14, mapk1, TNF, IL6, and other proteins were the main targets of RA combined with PRR in the treatment of osteoporosis. JUN protein is a family of protein kinases in the mitogen-activated protein kinase (MAPK) signal transduction cascade, and c-Jun is also an essential substance for activating transcriptional activator protein 1 (AP-1) [17]. Studies have shown that the increase of JUN protein level can initiate more AP-1, AP-1 can affect the apoptotic process of cells, promote apoptosis, and accelerate the differentiation of osteoclast precursor

cells into osteoclasts by regulating the expression of related genes[18]. In addition, it can also increase the activity of metalloproteinases to promote the degradation of collagen in human skin[19–20]. Src proteins are a class of non-receptor protein tyrosine kinases activated by various extracellular signaling molecules[21]. Many studies have shown that the high activation of Src protein is closely related to the occurrence and development of osteoporosis. Its inhibitors are expected to be a critical method for treating osteoporosis drugs[22]. AKT1 is a member of the protein kinase (B) family and is necessary for cell proliferation, cell growth, and differentiation in the body. The researchers found that mice with the AKT1 gene knocked out had skeletal muscle atrophy and impaired bone development. In recent years, TNF and IL6 factors have been considered the main factors regulating bone resorption under pathological conditions, and they have a close relationship with estrogen levels. Many experiments in vitro and in vivo have confirmed that IL-6 can elevate the formation of osteoclasts and increase bone resorption[23–24]. TNF indirectly activates mature osteoclasts by stimulating osteoblasts and inhibits the apoptosis of osteoclasts[25].

KEGG pathway enrichment analysis results showed that RA combined with PRR treatment of osteoporosis mainly involves PI3K-Akt, MAPK, TNF, Rap1, Toll-like receptors, HIF-1, Ras signaling pathways. PI3K/Akt pathway is closely related to osteogenesis and osteoclast pathways. The PI3K/Akt pathway can regulate the differentiation and apoptosis of osteoblasts and osteoclasts to maintain the balance of bone resorption and bone formation[26]. Decreasing the activity of caspases-9 through the PI3K-Akt pathway can weaken the absorptive capacity of osteoclasts and reduce the apoptosis caused by cell damage, thereby delaying the process of osteoporosis[27]. The MAPK pathway is directly involved in the regulation of bone metabolism. It has a complementary relationship with the PI3K/Akt pathway[28] and plays a vital role in promoting osteoblast growth and differentiation. MAPK signaling pathways mainly include extracellular regulated protein kinase (ERK) transduction pathway, Jun N-terminal kinase (JNK), ERK5/macrofilament mitin-activated protein kinase transduction pathway, and p38 signaling pathway. The JNK pathway mainly affects bone formation[29]. It has been shown that fucoidan promotes the osteogenesis of human bone marrow stem cells by increasing phosphorylation-induced BMP2 expression and stimulating the activation of ERK, JNK, and p38 signaling pathways[30]. The p38 pathway and ERK signaling pathway can promote the expression of osteoprotegerin, which is the key to maintaining the balance of bone metabolism[31]. Tumor necrosis factor (TNF) is the main cytokine regulating osteoclast activity and bone resorption[32]. Researchers isolated the glycoprotein osteocalcin (OPG) from TNF factors through fetal mouse gene sequencing experiments, which can be used as a soluble factor to regulate bone mass[33].

In addition, this study established a rat model of osteoporosis by removing the rat's ovaries. The mechanism of RA combined with PRR in the treatment of osteoporosis was further explored through experimental methods such as dual-energy X-ray absorption assay, enzyme-linked immunosorbent assay, and HE staining method. Experimental results showed that RA combined with PRR could promote bone formation and increase BMD. The experimental results showed that the bone formation marker BGP was significantly increased compared with the model group, and the levels of β-CTX, MSTN, and PIIINP were significantly decreased. It showed that RA combined with PRR could increase bone formation markers to

promote bone formation and have a therapeutic effect on osteoporosis. Pathological examination results showed that the bone tissue of the model group was disordered, and the bone mass was reduced. RA combined with PRR could significantly improve the microstructure of bone tissue in osteoporotic rats.

Conclusion

In summary, this article used network pharmacology technology combined with in vivo experimental verification to screen out the active ingredients, key targets, and signal pathways of RA combined with PRR in treating osteoporosis. This article analyzed the mechanism of RA combined with PRR in treating osteoporosis from the perspective of multiple targets, multiple pathways, and multiple components. It provided new research methods and a theoretical basis for guiding its clinical application.

Abbreviations

OP:osteoporosis; RA:Radix Astragali; PRR:Prepared Radix Rehmannia; TCMSP:Traditional Chinese Medicine Systems Pharmacology; OB:Oral Bioavailability; DL:Drug likeness; PPI: protein-protein interaction; GO:Gene ontology; KEGG:Kyoto Encyclopedia of Genes and Genomes.

Declarations

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Author contributions

Wenqian Kang performed the data analysis,wrote the first version of the manuscript ,Chunyu Liu processed the graph and the table in the manuscript. Yu Tang finalized the manuscript. Zhixin Geng and Jiahao Zhang collected the data. Li Ou and Peifeng Wei (corresponding author) conceived and coordinated the study. All authors read and approved the final manuscript.

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Availability of data and materials

All the data will be available upon motivated request to the corresponding author of the present paper.

Ethical approval and consent to participate

This study was conducted in agreement with the Declaration of Helsinki and its later amendments or comparable ethical standards. The Animal Ethics Committee of Shaanxi University of Traditional Chinese Medicine approved the protocol (ethics approval number: AEC-19-002).

Competing interests

The authors declare no conflict of interest.

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References

1. Feng JW. Clinical Study of Calcitonin and Bisphosphonates in the Treatment of Primary Osteoporosis [J]. Jilin Medical Journal. 2017; 38(3): 529–31.
2. Ma Z, Li X, Chen Y, Tang X, Gao Y, Wang H, et al. Comprehensive evaluation of the combined extracts of Epimedii Folium and Ligustri Lucidi Fructus for PMOP in ovariectomized rats based on MLP-ANN methods. J Ethnopharmacol. 2021; 268:1135–63. doi: 10.1016/j.jep.2020.113563.
3. Ma YZ, Wang YP, Liu Q, Li CL, Ma X, Wang YJ, et al. Chinese Guidelines for the Diagnosis and Treatment of Osteoporosis in the Elderly (2018) [J]. Chinese Journal of Gerontology. 2019; 39(11): 2557–75.
4. Wang BS, Bao TT, Ren FG, Chang SR, Li YH. Meta-analysis on the treatment of middle-aged and elderly patients with primary osteoporosis by tonifying kidney, invigorating spleen and activating blood circulation [J]. Chinese Journal of Osteoporosis. 2021; 27(6): 824–30.
5. Wang GY, Liu XL, Chen S, Zhang G. Research Progress of Osteoporosis Treated by Traditional Chinese Medicine [J]. Hunan Journal of Traditional Chinese Medicine. 2020; 36(6): 162–5. https://doi:10.16808/j.cnki.issn1003-7705.2020.06.064.
6. Guo HG, Xie XW, Gu YB, Li N, Li DP, Shi YL,et al. Correlation between Primary Osteoporosis and OPG/RANK/RANKL Signaling Pathway from TCM "Deficiency" Treatment [J]. Chinese Journal of Osteoporosis. 2021; 27(5): 742–6.

7. Ou L,Cheng HY, Zeng XH. The effect of astragalus combined with prepared rehmannia root in treating 29 postmenopausal osteoporosis cases[J]. Chinese Journal of Gerontology. 2012; 32:3990–1.
8. Hu N, Zhang XJ. Progress in Chemical Constituents and Pharmacological Action of Astragalus [J]. Chinese Medicine Information. 2021; 38(1):76–82. https://doi:10.19656/j.cnki.1002-2406.210118.
9. Li X, Wang N, Wei XL, Zhang ZL. Historical Evolution and Modern Research Progress of Rehmannia Charcoal and Rehmannia Charcoal Processing [J]. Chinese Journal of Traditional Chinese Medicine. 2012; 30(8): 1737–1739. https://doi:10.13193/j.archtcm.2012.08.43.lix.038.
10. Chen F, Zhang Y, Xu L, Liu Q, Kui J, Yang LJ, et al. Progress in the Treatment of Postmenopausal Osteoporosis with Chinese and Western Medicine [J]. Medical Herald. 2021; 40 (9): 1216–20.
11. Yan W, Wang ZH, Liu H\. Mechanism of Osteoporosis Caused by Imbalance of Bone Remodeling and Its Drug Treatment Strategy [J]. Chinese Journal of Tissue Engineering Research. 2020; 24(30): 4866–74.
12. Saad FA. Novel insights into the complex architecture of osteoporosis molecular genetics. Ann N Y Acad Sci. 2020; 1462(1):37–52. doi: 10.1111/nyas.14231.
13. Ding SQ, Yang FQ, Zhang W, Ma XQ. Experimental study on the treatment of osteoporosis in ovariectomized rats with kaempferol [J]. Chinese Journal of new drugs. 2014; 23(24): 2925–9 + 32.
14. Wang XF, Yang SC Lixin, Zhang Ziyu. Protective effect of kaempferol on promoting bone collagen production and reducing bone trabecular loss in ovariectomized rats [J]. Chinese Journal of clinical pharmacology. 2020; 36(10): 1302–5. https://doi:10.13699/j.cnki.1001-6821.2020.10.036.
15. Casado-Díaz A, Anter J, Dorado G, Quesada-Gómez JM. Effects of quercetin, a natural phenolic compound, in the differentiation of human mesenchymal stem cells (MSC) into adipocytes and osteoblasts. [J]. J Nutr Biochem. 2016; 32:151–62. https://doi: 10.1016/j.j.jnutbio.2016.03.005.
16. Bian W, Sun H, Liu K, Yang L, Zhang RH. Effects of quercetin on bone biomechanical properties and bone metabolism in osteoporosis rats [J]. Jilin traditional Chinese medicine. 2016. 36(8): 814–7. https://doi: 10.13463/j.cnki.jlzyy.2016.08.018.
17. Chen XJ, Liu J. Network pharmacological mechanism of Angelica dahurica in the treatment of acne [J]. Journal of Guangzhou University of traditional Chinese medicine. 2019; 36(10): 1624–31. https://doi: 10.13359/j.cnki.gzxbtcm.2019.10.026.
18. Yuan Z, Li Q, Luo S, Liu Z, Luo D, Zhang B,et al. PPARγ and Wnt Signaling in Adipogenic and Osteogenic Differentiation of Mesenchymal Stem Cells. Curr Stem Cell Res Ther. 2016; 11(3):216–25. doi: 10.2174/1574888x10666150519093429.
19. Liu P, Yang RL, Su H, Li LL, Song JW, Lu N, et al. Infrared affects the expression of c-jun, type I and type III collagen in human skin fibroblasts [J]. Journal of Southern Medical University. 2016; 36(2): 163–9.
20. Chen GM, Wang L, Du P, Li ZP, Shen HY.et al. Protective effect of Ophiopogon japonicus polysaccharide on human skin fibroblasts damaged by UVB [J]. China tissue engineering research. 2017; 21(32): 5183–8.

21. Guo SM, Lin YP. The role of c-Src protein in osteoporosis [J]. Chinese Journal of orthopedics and traumatology. 2014; 22(8): 72–5.
22. Machida K, Mayer BJ. The SH2 domain: versatile signaling module and pharmaceutical target. Biochim Biophys Acta. 2005; 1747(1):1–25. doi: 10.1016/j.bbapap.2004.10.005.
23. Peng XD, Xu PZ, Chen ML, Hahn-Windgassen A, Skeen J, Jacobs J, et al. Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. Genes Dev. 2003; 17(11):1352–65. doi: 10.1101/gad.1089403.
24. Zhou PQ, Shen L, Du JY, Yang YP, Xie J, Gao L, et al. Postmenopausal osteoporosis and serum IL-6, TNF- α Clinical study on concentration relationship [J]. Chinese Journal of osteoporosis. 2001 (1): 5-7 + 12.
25. Jiang Ling. Role of cytokines in regulating bone resorption [J]. Foreign medicine (Geriatrics). 1995(5): 198-201.
26. Chen YH, Gong ZQ, Cui L. The role of PI3K / Akt signaling pathway in the pathological process of osteoporosis [J]. Chinese Journal of osteoporosis. 2015; 21(3): 356–60.
27. Qiang SL, Liu T, Jia YL, Xu KW, Yu HY, Jiang YW, et al. Study on the regulation of Bushen Guben Recipe on the expression of PI3K / Akt / mTOR signal pathway in rat osteoporosis model [J]. Chinese Journal of osteoporosis. 2020; 26(7): 983–7.
28. Fan WB, Bao NR, Zhao JN. The role of PI3K / Akt signaling pathway in bone metabolism [J]. Chinese Journal of Orthopaedic Surgery. 2013; 21(19): 1958–62.
29. Chen MY, Xie JY. Research progress on the pathogenesis of osteoporosis [J]. Journal of Xiangnan University (Medical Edition). 2018; 20(1): 63–6. doi: 10.16500/j.cnki.1673-498x.2018.01.022.
30. Kim BS, Kang HJ, Park JY, Lee J. Fucoidan promotes osteoblast differentiation via JNK- and ERK-dependent BMP2-Smad 1/5/8 signaling in human mesenchymal stem cells. Exp Mol Med. 2015; 47(1):e-128. doi: 10.1038/emm.2014.95.
31. Matsuguchi T, Chiba N, Bandow K, Kakimoto K, Masuda A, Ohnishi T. JNK activity is essential for Atf4 expression and late-stage osteoblast differentiation. J Bone Miner Res. 2009; 24(3):398–410. doi: 10.1359/jbmr.081107.
32. Tian AX, Ma JX, Ma XL. Osteoporosis associated molecular signaling pathway and its clinical application [J]. Chinese Journal of osteoporosis and bone mineral diseases. 2019; 12(3): 282–91.
33. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Lüthy R, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell. 1997; 89(2):309–19. https://doi: 10.1016/s0092-8674(00)80209-3.

Figures

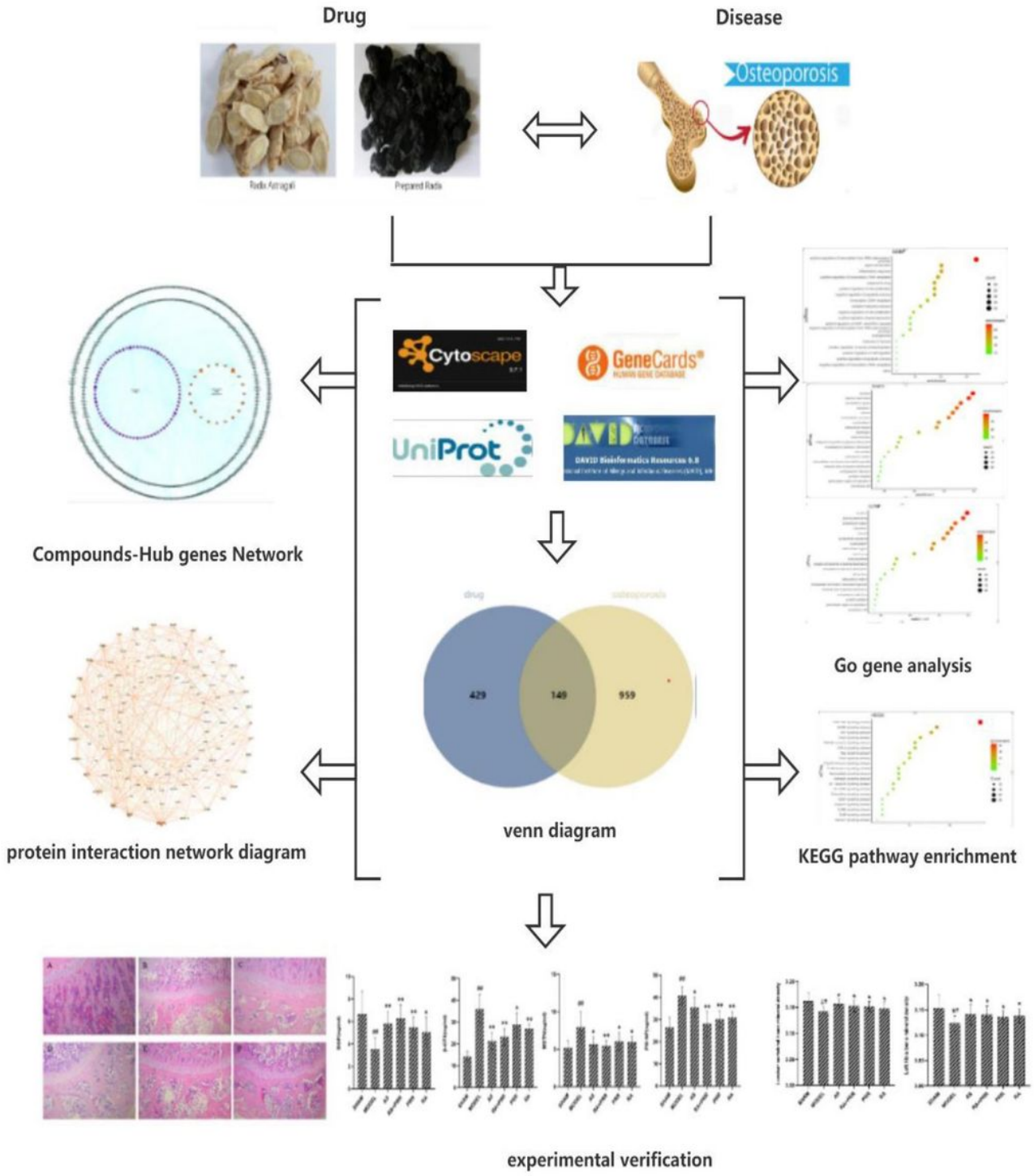

Figure 1

A detailed flow chart of the study

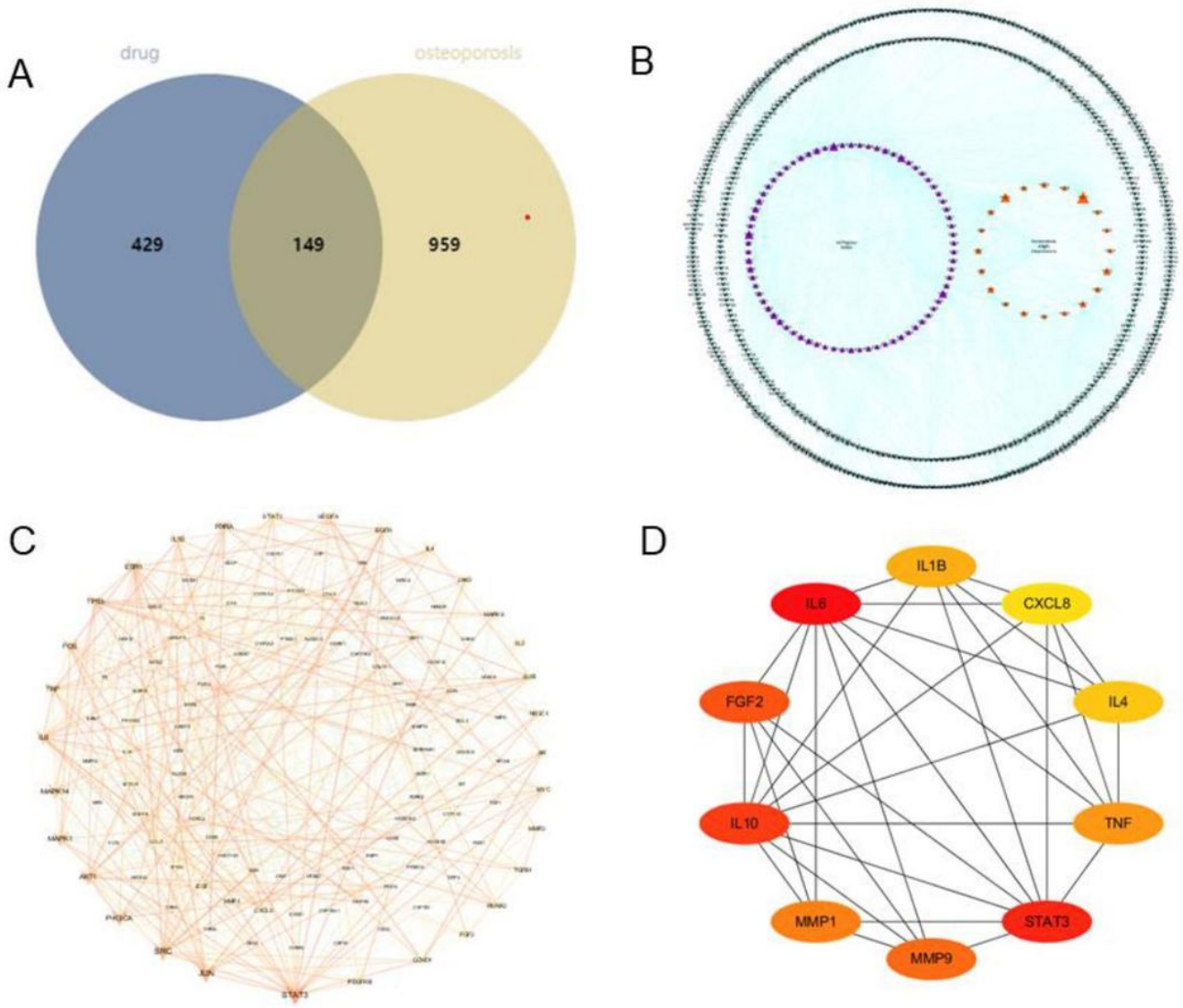

Figure 2

RA-PRR and OP core target network diagram. (A)Venn diagram of RA-PRR and OP intersection targets. (B)Ingredient-target network of herbal pair RA-PRR. (C)PPI network of potential targets. (D)core target protein map.

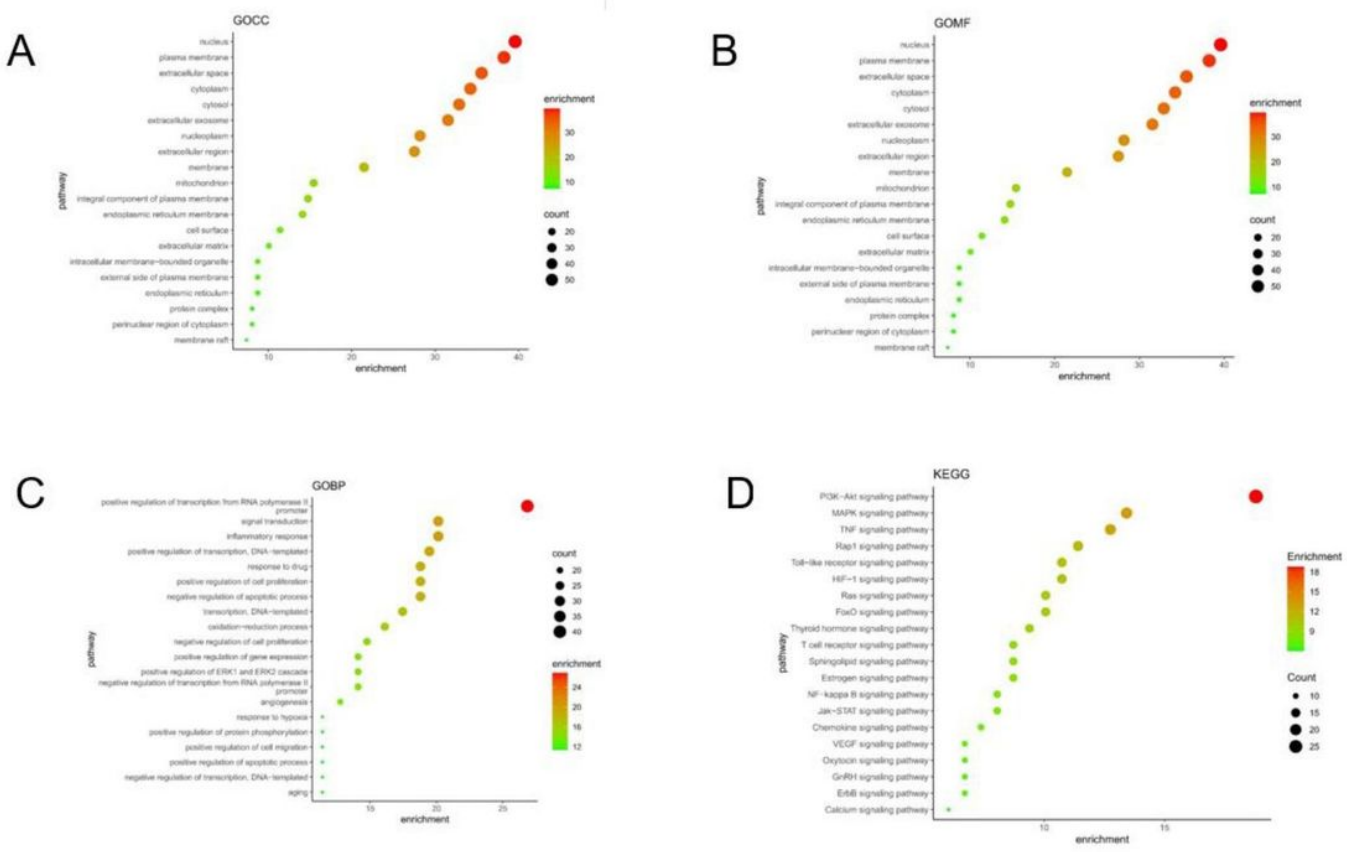

Figure 3

GO analysis and KEGG pathway diagram of RA combined with PRR in the treatment of OP. (A–C) GO analysis bubble chart. (D) Bubble Diagram of KEGG enrichment analysis.

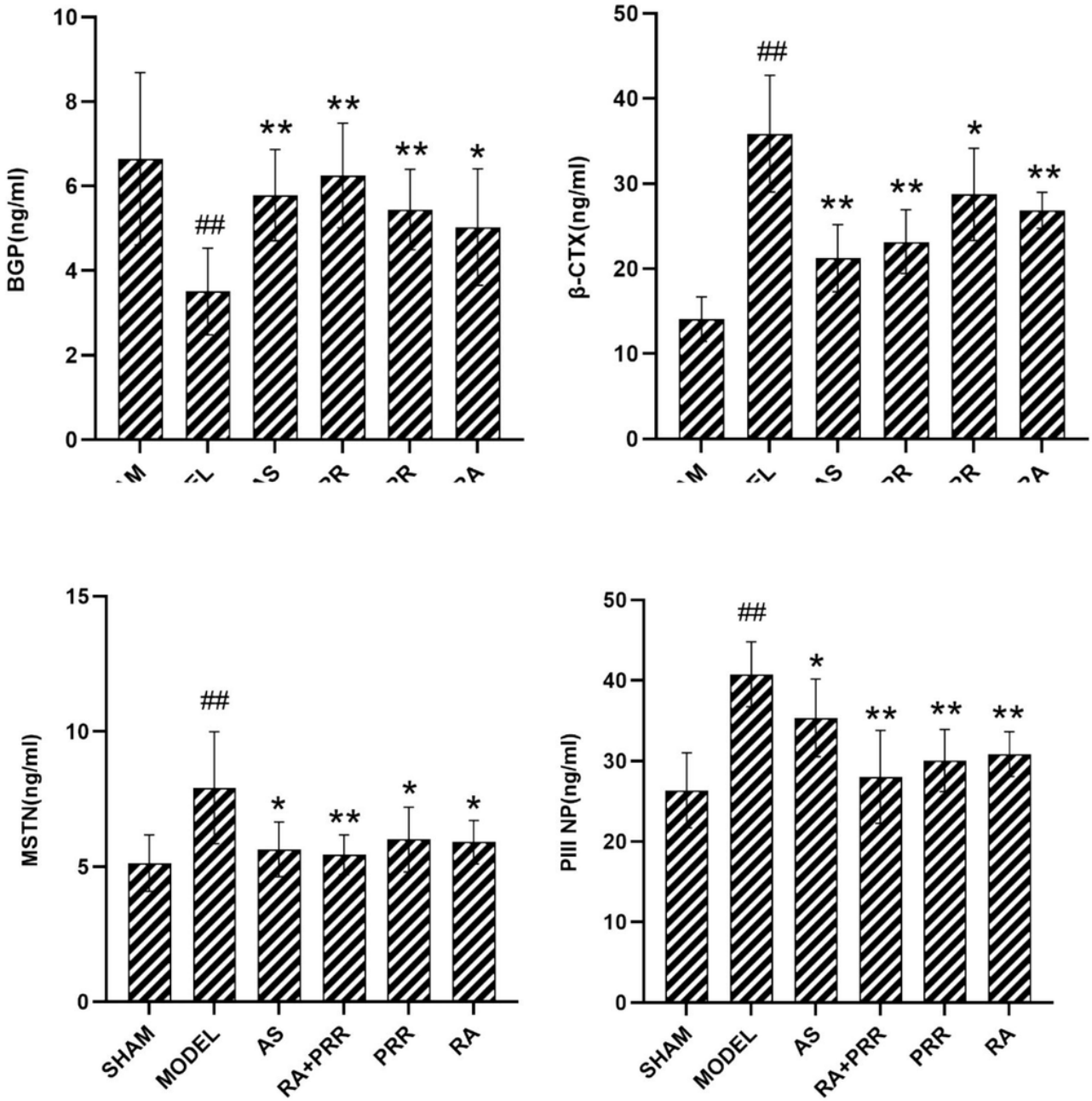

Figure 4

Effect of RA combined with PRR on BGP、β-CTX、MSTN、PIIINP in serum Note:vs the sham operated group,#P<0.05,##P<0.01;vs the model group,*P<0.05,**P<0.01.

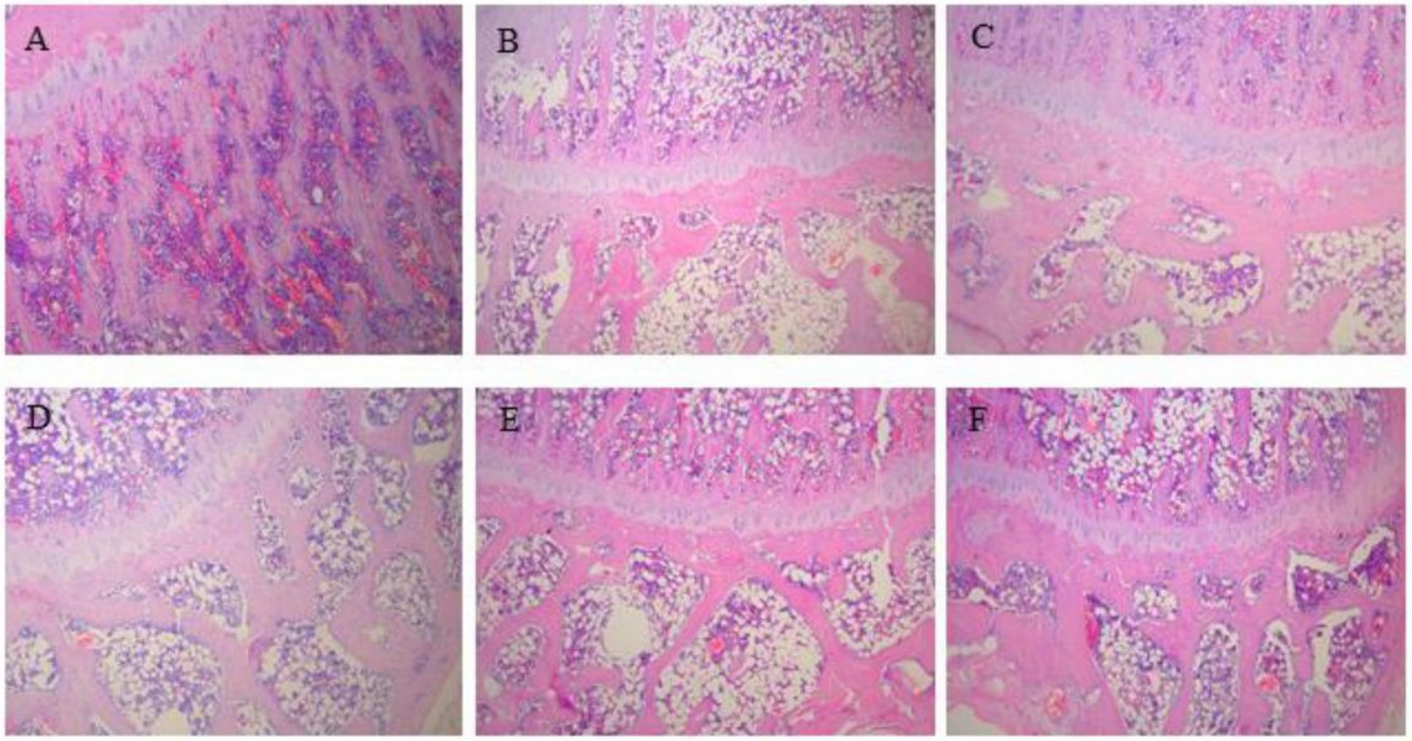

Figure 5

Effect of RA combined with PRR on osseous pathologic changes in ovariectomized rats (HE ×40). (A)sham; (B)model; (C)model+AS; (D)model+RA; (E) model+PRR; (F)model+RA+PRR.

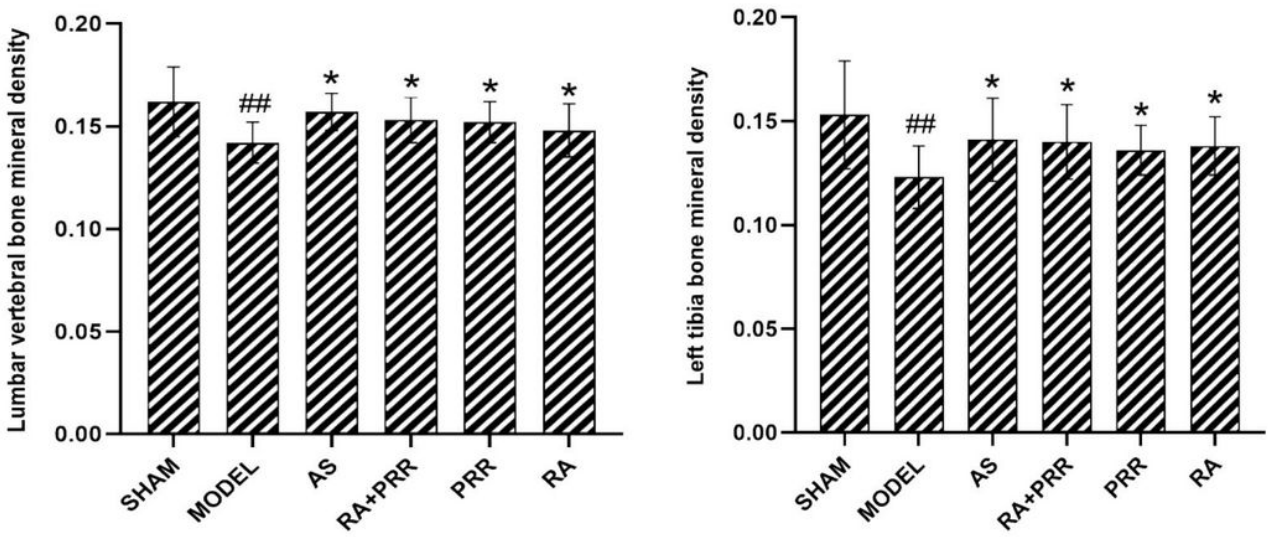

Figure 6

After 12 weeks of drug intervention. n=10, vs the sham operated group, #P<0.05, ##P<0.01; vs the model group, *P<0.05, **P<0.01.

Supplementary Files

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- TableS1.docx