Verification of the therapeutic effects and neural mechanism of Bushen Zhuangjin Decoction in the treatment of knee osteoarthritis

Xue Tan  
Fujian University of Traditional Chinese Medicine

Danhao Zheng  
University of the Chinese Academy of Sciences

Qing Lin  
Fujian University of Traditional Chinese Medicine

Lili Wang  
Fujian University of Traditional Chinese Medicine

Zaishi Zhu  
Fujian University of Traditional Chinese Medicine

Yanfeng Huang  
Fujian University of Traditional Chinese Medicine

Yihui Zeng  
Fujian University of Traditional Chinese Medicine

Min Mao  
Fujian University of Traditional Chinese Medicine

Zhouping Yi  
Fujian University of Traditional Chinese Medicine

Linglong Liu  
Fujian University of Traditional Chinese Medicine

Dezun Ma  
Fujian University of Traditional Chinese Medicine

Jie Wang  
State Key Laboratory of Magnetic Resonance and Atomic and Molecular Physics

Xihai Li  
Fujian University of Traditional Chinese Medicine  
https://orcid.org/0000-0003-2847-2168

Keywords: Bushen Zhuangjin Decoction, Knee osteoarthritis, Pain, Neuropeptides, Functional magnetic resonance imaging
Abstract

Chronic pain is the principal clinical manifestation of knee osteoarthritis (KOA) and an essential indicator of the diagnosis and treatment effect. Changes in brain functional activity are related with chronic pain in KOA. Bushen Zhuangjin Decoction (BZD) has been proved to reduce inflammation of arthritis, improve cartilage degeneration and analgesia, but whether it plays a role through the change of brain function activity is not clear. Here, three experiments were performed: (1) network pharmacology evaluation to discover the potential targets of BZD to relieve pain in KOA; (2) verification of the therapeutic effects of BZD treatment on KOA pain with histomorphology, behavioral assessments, suspension chip analysis, and ultra-performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) assays; and (3) functional magnetic resonance imaging to explore the effects of BZD treatment on brain function associated to KOA. The analgesic effect of BZD on KOA was found to be related to the neurotransmitters of pain signals through network pharmacology and the therapeutic effect of BZD on KOA pain was verified in vivo, and related to neuropeptides and neurotransmitters. Functional magnetic resonance imaging showed that BZD treatment could reverse the regional homogeneity/amplitude of low-frequency fluctuation analysis in pain-related brain regions of KOA, suggesting that the analgesic mechanism of BZD is related to neural regulation. This study confirmed the key position of pain-related neuromodulation mechanisms in the analgesic therapy of BZD and provide a theoretical basis for the treatment of KOA pain with BZD as a traditional Chinese medical.

Introduction

Knee osteoarthritis (KOA) is a common inflammatory degenerative joint disorder disease characterised through cartilage degeneration and subchondral bone remodeling. Chronic pain is the main symptom in patients seeking medical treatment and an important cause of disability in osteoarthritis [1, 2]. KOA pain is caused by the interaction of mechanical stress changes, cartilage, subchondral bone, or meniscus injury, peripheral or central nervous system sensitization, and other factors. Changes in mechanical stress led to axon growth of subchondral bone nerves and sensory and sympathetic nerve invasion [3]. Nerve-related factors act as messengers that bind to free nerve endings, changing afferent sensitivity and causing pain. Continuous input of peripheral stimulus signals causes spontaneous neuronal activity, leading to central sensitization [4]. Subchondral bone remodeling is the structural groundwork of osteoarthritis pain and neuromodulation is the deciding aspect of pain. The nervous system is essential for maintaining joint homeostasis and tissue repair [5]. The pain factor 5-HT is involved in the generation and persistence of pain by activating related ion channels and cytokines and their receptors [6]. Neuropeptides, such as substance P (SP), calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY) and tyrosine hydroxylase (TH), are involved in the mechanisms of pain appreciation to various degrees. They play a role in transmitting pain signals, regulating bone homeostasis, and promoting inflammatory responses in KOA [7]. CGRP and NPY can also promote angiogenesis. Clinical research has determined that patients with KOA have whole brain and regional gray matter loss [8]. Changes in functional activity in the cerebral cortex, hippocampus, hypothalamus and cingulate nucleus are associated with KOA pain.
Bushen Zhuangjin Decoction (BZD) has been used to deal with osteoarthritis in China for a long time. Clinical researchs have proven that BZD can efficaciously relieve pain and improving the severity of osteoarthritis. The potential mechanism of BZD in improving KOA pain may be 1) inhibition of inflammation [9], 2) inhibition of chondrocyte apoptosis [10], 3) regulation of skeletal signaling pathways, and 4) regulation chondrocyte cycle regulation factors and improves chondrocyte vitality [11]. However, whether BZD affects the changes of brain functional activity, neurotransmitter transmission and pain in knee osteoarthritis has not been reported. Therefore, we hypothesized that BZD could improve brain functional activity by regulating the transmission of neural signals, thereby reducing pain.

Network pharmacology is an integrative biology research method. It combines a range of disciplines to assemble the relationship between drugs, ingredient, target, and disease, and opens up a new viewpoint for the model of drug and disease research [12]. Network pharmacology in the study of KOA is mainly reflected in the sorting and analysis of disease data, which significantly contributes to explaining the pathogenesis and diagnosis of KOA [13].

The purpose of this study was to look at the impact of BZD on the therapy of KOA pain and its neuromodulation. Using a network pharmacological analysis, we explored the potential targets involved in relieving KOA pain by BZD. The therapeutic effect of BZD on KOA pain was verified in a traditional KOA animal model using morphology, behavioral tests, and suspension chip detection. Finally, functional magnetic resonance imaging (fMRI) was used to explore the correlation between the BZD treatment effect and brain regions and to clarify the neuromodulation mechanism of BZD and pain. We accept as true with the effects of this study will make contributions to a higher appreciation of the pathogenesis of pain in KOA and provide further theoretical basis for the clinical application of BZD.

**Materials And Methods**

**Network pharmacology analysis to explore the potential targets of BZD therapy in KOA**

**Active compounds of BZD**

The compounds of *Rehmanniae Radix Praeparata, Comus Officinalis, Dipsaci Radix, Eucommiae Cortex, Eleutherococcus gracilistylus, Angelicae Sinensis Radix, Paeoniae Radix Alba, Poria Cocos, Citri Reticulatae Pericarpium Viride*, and *Achyranthis Bidentatae Radix* in BZD were obtained from the traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, tcmspw.com/tcmsp.php). Biological properties of drugs including molecular weight, oral bioavailability (OB) and drug similarity (DL) can be accrued from the TCMSP database. OB and DL were used as indicators for screening effective compounds [14]. In this study, the following screening stipulations were used to reap the active compounds of BZD: OB ≥ 30%, DL ≥ 0.18 [15]. And potential targets of BZD can be also collected from the TCMSP platform.
Active compounds and predicted subchondral bone targets of BZD treatment in KOA

Genes for human osteoarthritis were gathered from the following databases: GeneCards (https://www.genecards.org/), Online Mendelian Inheritance in Man (OMIM, https://www.omim.org/), PharmGkb (https://www.pharmgkb.org/), Therapeutic Target Database (https://bidd.nus.edu.sg/bidd-databases/TTD/TTD.asp), and DrugBank (https://www.DrugBank.ca) by using "osteoarthritis" as a keyword [16]. In order to make the results more comprehensive and reliable, these five databases are combined here. Then, this list was combined with subchondral bone targets collected from the GSE51588 dataset, which was downloaded from the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/) with osteoarthritis and subchondral bone as keywords and human was selected as the category [17]. The intersection is the differential gene of subchondral bone in human osteoarthritis. The intersection of genes that overlapped with the potential targets of BZD was considered predicted subchondral bone targets of BZD treatment in KOA. The traditional Chinese medicine botanical agents acting on these predicted targets were considered the active components of BZD.

Protein-protein interaction network construction and pathway enrichment analysis

The STRING (https://cn.string-db.org/) database was used to construct a protein-protein interaction (PPI) network to analyze the functional interactions between proteins [18]. In this study, the PPI protein interaction network removing the discrete targets can be obtained by selecting the discrete points in the hidden diagram in the settings. R software program permits bioinformatics analysis and visualization of results. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis visualizes data by using the three R software installation packages, “enrichplot,” “DOSE,” and “clusterProfiler”. P-value cutoff = 0.05 and q-value cutoff = 0.05 were used to screen the KEGG results with significant differences. Then, the subchondral bone-associated neural signaling pathways of KOA pain were screened using the KEGG database (https://www.kegg.jp/).

Verification of the therapeutic effects of BZD in KOA in vivo

Animals

All animal remedy strategies observed the suggestions of the Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine (Approval Number: 2019062). Forty-five healthful male Sprague–Dawley rats (8 weeks old, weighing 180–220 g each) were purchased from Hangzhou Medical College (Hangzhou, Zhejiang, China). The rats were raised at 23°C room temperature and lights (12:12 h light-dark cycle) with advert libitum food and water. The animals have been allowed adaptive feeding for one week earlier than the begin of the experiments.
Koa Rat Model

Animals were randomly assigned to sham group (n = 15), KOA model group (n = 15), and KOA + BZD treatment group (n = 15). The KOA group adopted the modified Hulth method to establish model [19]. The rats were anesthetized with isoflurane (1.5–2.0%) and disinfected with skin application. Briefly, 30 rats underwent a patellar incision on the medical side of the knee [20]. The medial collateral ligament and anterior cruciate ligament were severed, and the medial meniscus used to be resected. Once no lively bleeding was observed, the incision was sutured by way of layer. In the sham group, solely the knee cavity and sutured incision were exposed. Two weeks after surgery, 15 KOA rats were randomly selected for treatment with BZD (10.5 g/kg, once/d, intragastrical). The intervention dose of BZD was transformed into the intervention dose of rats in accordance to the equivalent dose of human and rat. Sham group and KOA group were also given the equal dose of 0.9% normal saline intragastric administration.

Pain-related Behavioral Assessments

The paw withdrawal threshold (PWT) and paw withdrawal latency (PWL) tests were used as pain indicators in accordance to previous research [21, 22]. PWT was measured using a digital force meter (YLS-3E, Yima Optoelec Co., Ltd., China) with a force range of 0–2000 g and a resolution of 0.1 g. A continuous elevation force was applied to the hind paw until the retraction reflex occurred. The force intensity was recorded as the PWT. The rats were positioned in a glass chamber to acclimate for 15 min earlier than carrying out PWL. PWL measurements were performed on animals using a heat pain stimulator (BME-410C, American Institute of Biomedical Engineering) with a 12 V/10 W halogen lamp. The stimulation temperatures were 45°C–65°C, with a time accuracy of 10 ms and a cutoff time of 20 s. The time of the withdrawal response was recorded as the PWL. Before each test, the heat source was cooled until it returned to room temperature, and the glass chamber was kept dry to prevent urine from affecting the test results.

Hematoxylin & Eosin, Safranin O-fast Green, And Masson Staining

After 12 weeks of intervention, the tibial plateau was fixed in 4% paraformaldehyde solution for 48 h and decalcified with 10% ethylenediamine tetraacetic acid disodium salt (EDTA-2NA) for 8 weeks. After 8 weeks, the extra ligaments and other tissues were pruned and then paraffin embedded. The paraffin-embedded tissue was fixed on a slicer, cut into 8-µm thick slices, and blanched flat in 37°C water. The slices were positioned on slides and dried in an incubator at 55°C. Then, xylene transparent treatment and dewaxing were performed with high to low concentrations of anhydrous ethanol. Hematoxylin & eosin, safranin O-fast green, and Masson staining were carried out in accordance to the directions of the package (Solarbio, China). Light microscopy (DM4000B, Leica, Germany) was used to study cartilage and subchondral bone morphology and structure.
Detection Of Inflammatory Factors In Rat Serum By Suspension Chip Analysis

After intervention, the animals were anesthetized (isoflurane, 1.5–2%), blood was accrued from the inferior vena cava, and the serum was used to prepare in a centrifuge (1000 g, 15 min, 4°C, then 1000 g, 10 min, 4°C) for suspension chip analysis. The serum contents of 23 inflammatory factors were assessed with a multiplex assay package (Bio-Plex Pro Rat Cytokine 23-Plex Kit, Bio-Rad, USA). The results were detected using the corrected Bio-Plex 100, 200, and 3D systems.

Detection Of Neurotransmitters In Rat Serum

After sampling, the venous blood was rested for 1 h at room temperature and centrifuged (3000 g, 10 min, 4°C), and the supernatant was accrued for similarly centrifugation (12000 g, 10 min, 4°C) for UPLC-MS/MS Analysis. UPLC separation was used to carry out using the usage of ExionLC System geared up with a Waters ACQUITY UPLC HSS T3 (100 × 2.1 mm, 1.8 µm). For the mobile phase, phase A was 0.1% formic acid and 1 mM/L ammonium formate in water, and phase B was acetonitrile. The column temperature was 40°C. The auto-sampler temperature was set at 4°C, and the injection volume was 1 µL. The AB Sciex QTrap 6500 + mass spectrometer was used for the assay. Typical ion source parameters were: IonSpray voltage: +5000 V, curtain gas: 35 psi, temperature: 400°C, ion source gas 1: 60 psi, and ion source gas 2: 60 psi. Skyline software was employed for multiple reaction monitoring (MRM) data processing.

Enzyme-linked Immunosorbent Assay

Blood was used to gather from the vena cava, and serum was prepared in a centrifuge (10000 g, 30 min, 4°C). The upper serum was placed in a 0.5-mL Eppendorf tube and a − 80°C fridge for enzyme-linked immunosorbent assay (ELISA). SP, CGRP, NPY, and TH were examined with ELISA kits (TSZ, USA) in accordance to the manufacturer's instructions. All measurements had been taken by means of skilled technicians who have been blinded to the therapy groups.

Western Blot Analyses

Western blot analyses
Standard western blot analysis was used to detect the levels of related proteins, including collagen II (Col-II), matrix metalloproteinase-3 (MMP-3), MMP-9, and MMP-13 in cartilage and receptor activator of nuclear factor-kappa B ligand (RANKL)/osteoprotegerin (OPG), alkaline phosphatase (ALP), tartrate-resistant acid phosphatase (TRACP), SP, CGRP, NPY, TH, 5-HT₃, and VEGFA in the subchondral bone. The primary antibodies were as follows: Col-II, MMP-9, ALP, TH, 5-HT₃ and VEGFA (1:1000, Abcam, USA), MMP-3 (1:2000, Abcam), MMP-13 (1:2000, Proteintech, China), RANKL (1:1000, Proteintech), OPG (1:300,
Abcam), TRACP (1:4000, Abcam), SP (1:2000, Sigma–Aldrich, USA), CGRP and NPY (1:1000, CST, USA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10000, Abcam, USA) and β-actin (1:1000, Abcam, USA). The membrane was incubated with the corresponding secondary antibody (goat anti-rabbit IgG, 1:5000, Bioss, China) conjugated with HRP for 1 h at room temperature. Immunoreactive proteins were visualized the usage of an enhanced chemiluminescence western assay kit (Thermo Fisher Scientific, USA). Image Lab software program was used to quantify the density of each strip.

Exploration Of The Potential Neuromodulation Mechanism Of Bzd With Resting-state Fmri

The rats were anesthetized (with 1.5–2.0% isoflurane) and constant to a headrest with a built-in coil and physique tube. Isoflurane (0.5–1.0%) was delivered in a 30% O₂/70% N₂ mixture through a nose cone. The whole system was positioned in a magnet. Respiratory rate and rectal temperature were closely monitored throughout the procedure. The animal’s core temperature was maintained at 37°C ± 0.5°C the use of a cycling heated bed. The T2W structural image acquisition was implemented in a 9.4 T MRI scanner (Biospec 9.4, Bruker Medizenk, Germany), and an orthogonal surface coil with a diameter of 35 mm was utilized. T2 weighted imaging was used to swiftly obtained with a relaxation enhancement sequence with the following parameters: repetition time (TR) = 2500 ms, echo time (TE) = 33 ms, field of view (FOV) = 3.2 × 3.2 cm, averages = 4, slice number = 21, slice thickness = 1.0 mm, matrix size = 256 × 256, bandwidth = 326084 Hz, echo spacing (ESP) = 10000 ms, refocusing angle = 180°, excitation angle = 90°, echo train length (ETL) = 8, and k-zero = 3.

All resting-state fMRI statistics used AFNI (National Institutes of Health, USA), the Advanced Normalization Tools (ANTs) (stnava.github.io/ANTs/), and FMRIB’s Software Library (FSL) for processing. Processing of the T1-weighted image included: 1) “N4BiasCorrection” and “denoise image” commands to perform field deflection correction and image denoising, respectively; 2) manually removing the skull and scalp tissues of the image; and 3) registering the preprocessed images to the standard sigma template and partitioning the image [23]. The preprocessing of resting-state fMRI included the following steps: first, slice-timing correction and despike were carried out. Then, motion correction was used to limit the artifacts caused by head movement. Rigid registration of the fMRI and T1W images was carried out with the ANTs tool, and then time filtering was carried out at 0.01–0.1 Hz. Finally, the preprocessed resting-state fMRI data were normalized to sigma templates, regional homogeneity (ReHo) indicators were calculated, and amplitude of low-frequency fluctuation (ALFF) and functional connection matrices were calculated after smooth processing. The command “3dttest ++” in AFNI was used to test the ReHo/ALFF/functional connectivity indicators in a two-sample T test between two pairs (P < 0.05, cluster size ≥ 10 voxels).

Statistical Analyses
SPSS software (v.25.0; IBM, USA) was used for statistical analysis of the experimental data, and all values obtained from the experimental results are expressed as mean ± standard deviation. For normally distributed samples, one-way analysis of variance was used to compare groups. When the samples did not follow a normal distribution, a non-parametric test was used to compare the differences between groups. P-values of < 0.05 were considered statistically significant.

Results

Potential targets of BZD in the therapy of KOA

BZD contains 86 active ingredients, and 240 potential targets according to TCMSP database. Additionally, there were 1876 human genes of osteoarthritis, which were combined by removing duplicate genes from five disease databases, including 1835 genes in GeneCards database, 6 genes in OMIM database, 9 genes in PharmGKB database, 23 genes in Therapeutic Target database and 43 genes in DrugBank database. Combining the results of these databases and removing duplicate genes yielded 1,876 genetic targets (Fig. S1A). The database GSE51588 contains 4531 subchondral bone gene targets for KOA (Fig. S1B). There were 451 differentially expressed genes in the subchondral bone of KOA from the intersection of the above six disease databases (Fig. S1C). There were 36 subchondral bone predicted targets, and seven active compounds acted on these targets in BZD treatment of the subchondral bone of KOA, recognized through combining 451 subchondral bone targets with BZD targets in KOA (Fig. 1A). The predicted targets for BZD treatment in KOA subchondral bone included VEGFA, MMP-9, SELE, and MAPK1. The active components acting on these targets included catechin, naringin, β-carotene, quercetin, kaempferol, and β-sitosterol. 36 subchondral bone predicted targets were imported into STRING for PPI network analysis (Fig. S1D). VEGFA and MMP-9 play essential roles in the PPI. KEGG enrichment analysis of the predicted targets revealed 61 signaling pathways, including tumor-related, immune, cardiovascular, neurological, endocrine, and signal transduction pathways. Among these, the serotonergic synaptic pathway was associated with KOA pain (Fig. 1B). Additionally, 5-HT was identified as a key neurotransmitter in pain.

Validation Of The Therapeutic Effect Of Bzd

In vivo verification in the KOA rat model

Our morphological experiments confirmed the pathological effects of KOA and the therapeutic effects of BZD. KOA induces degeneration of the cartilage and remodeling of subchondral bone in the tibial plateau. Histopathological staining showed attenuation of the hyaline cartilage layer, a disordered arrangement of chondrocytes, and blurred and discontinuous tidal lines. Trabecular bone thinning and sparsely structure occurred, and new bone appeared (Fig. 2A). Regarding biomarkers of cartilage matrix degradation, the level of Col-II in the cartilage was significantly decreased in KOA, which was significantly increased by BZD treatment (Fig. 2B & D). Proteins associated with cartilage inflammation, including MMP-3, MMP-9,
and MMP-13, were upregulated in KOA and were significantly inhibited by BZD (Fig. 2B & C and 2E–G). For biomarkers of bone homeostasis, the bone resorption biomarkers RANKL/OPG and TRACP and the bone formation biomarker ALP in subchondral bone showed the same trend, and their levels were notably increased in KOA and significantly decreased by BZD (Fig. 2H–K). KOA significantly reduced the PWT and PWL, which were increased with BZD treatment, verifying the analgesic effects of BZD (Fig. 2L & M). At the cease of the intervention, there was no significant difference in body weight among the three groups (Fig. S3).

These results confirmed the successful establishment of a rat model of KOA, and the model showed sensitivity to pain. BZD treatment has been proven effective, but not complete recovery.

**In vivo validation of serum inflammatory factors and neurotransmitters by BZD**

Inflammatory factors and neurotransmitters act as messengers that carry pain signals through the body. The transmission of pain signals and the therapeutic effect of BZD were evaluated by detecting inflammatory factors and neurotransmitters in serum. Analysis of inflammatory factors showed that IL-1α, IL-5, IL-12, IL-17A, RANTES, tumor necrosis factor-α (TNF-α), and M-CSF significantly increased in KOA and were decreased by BZD (Fig. 3A–G). The serum IL-13 level was significantly decreased in KOA, but BZD significantly increased it (Fig. 3H). The levels of G-CSF, IL-7, IL-18, MCP-1, and MIP-3α in serum were increased by KOA and were inhibited by BZD (Fig. S4A–E). KOA decreased the content of IL-4 in serum, while BZD increased it (Fig. S4F). Neurotransmitters play an important role in the pain transmission, which can be evaluated by analyzing their expression levels in serum. The levels of 4-Aminobutyric acid (GABA), epinephrine (E), glutathione (GSH), kynurenine (Kyn), methionine (Met) and vanillylmandelic acid (VMA) in serum were significantly decreased by KOA and significantly increased by BZD (Fig. 3I-N). Meanwhile, the levels of tryptamine (TrpA), tyramine (TyrA), spermidine (Spd) and β-alanine (BALa) in serum were significantly increased by KOA and significantly decreased by BZD (Fig. 3O-R). Serum serotonin (5-HT), L-glutamic acid (Glu), 5-hydroxyindoleacetic acid (5-HIAA), histamine (Hist), 5-hydroxytryptophan (5-HTP), acetylcholine chloride (Ach-Cl), L-alanine (Ala), ornithine (Orn), L-lysine (Lys), L-asparagine (Asn) and spermine (Spm) levels were upregulated by KOA and downregulated by BZD (Fig. S4G-Q). The level of L (+)-arginine (Arg) in serum was downregulated by KOA and upregulated by BZD (Fig. S4R).

The therapeutic effect of BZD was confirmed based on **in vivo** validation. BZD can significantly reverse the pathological changes of KOA in animal models, but it did not result in complete recovery. BZD inhibits the transmission of pain signaling molecules and improves KOA pain.

**The Potential Mechanism Of Bzd In Treating Pain**

SP, CGRP, NPY, and TH modulate KOA pain. 5-HT is a neurotransmitter widely distributed in the peripheral and central systems, and its role in regulating pain depends on the receptor type. 5-HT binds to 5-HT₃ receptors to cause nerve excitation and transmit pain signals. VEGFA promotes angiogenesis and
exacerbates KOA pain. We discovered that in KOA, the levels of these serum neuropeptides are considerably elevated. In contrast, BZD appreciably decreased the expression of serum SP and TH. CGRP and NPY showed the same trend but no significant differences (Fig. 4A–D). Additionally, we recognized the expression of these proteins in the subchondral bone. The results were similar: in KOA, the expression of these proteins (SP, CGRP, NPY, TH, VEGFA, and 5-HT₃) was significantly upregulated, and BZD treatment significantly reversed this upregulation (Fig. 4E–M). Therefore, the therapeutic effect of BZD plays a neuroregulatory analgesic role.

**Bzd Relieves Koa-related Pain By Modulating Neural Mechanisms**

ReHo is primarily based on the similarity of hemodynamics of every voxel in a functional cluster and the dynamic synchronization of voxels in the equal cluster, through calculating the Kendall harmony coefficient between voxels and adjoining voxels in the time series. ALFF is a approach of calculating the square root of the power spectrum in the low frequency range (0.01 ~ 0.08 Hz) to learn about the changes of local spontaneous brain activity. ReHo and ALFF reflect the local synchronization and temporal consistency of resting brain activity and can be used as biomarkers to assess changes in brain function [24, 25]. The resting-state fMRI results showed that KOA treatment increased the ReHo of the cortex, hypothalamus, and hippocampus but significantly inhibited that of the striatum (Fig. 5A and Table 1). BZD intervention can alter the above pathological changes of KOA and increase the ReHo of striatum and insular cortex (Fig. 5B and Table 1). Furthermore, the ALFF of the insular cortex was increased in KOA but was inhibited in the hypothalamus, hippocampus, and cingulate gyrus (Fig. 5C and Table 2), which were all reversed by BZD treatment (Fig. 5D and Table 2). Therefore, BZD plays a role in treating KOA by regulating the activities of the cortex, hypothalamus, and hippocampus. Above consequences endorse that BZD performs an analgesic function thru a neuroregulatory mechanism.
Table 1  
ReHo changes in brain regions induce by BZD in KOA rats.

<table>
<thead>
<tr>
<th>ROI</th>
<th>NZcount</th>
<th>Min_T</th>
<th>Max_T</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sham vs. KOA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right insular cortex 2</td>
<td>136</td>
<td>-3.962</td>
<td>3.299</td>
</tr>
<tr>
<td>Left dorsal hippocampus</td>
<td>100</td>
<td>-4.492</td>
<td>3.223</td>
</tr>
<tr>
<td>Right auditory cortex</td>
<td>98</td>
<td>-3.679</td>
<td>4.132</td>
</tr>
<tr>
<td>Right dorsal hippocampus</td>
<td>79</td>
<td>-3.449</td>
<td>0</td>
</tr>
<tr>
<td>Left dorsal striatum</td>
<td>78</td>
<td>-2.984</td>
<td>3.367</td>
</tr>
<tr>
<td>Right insular cortex</td>
<td>77</td>
<td>-3.306</td>
<td>0</td>
</tr>
<tr>
<td>Right auditory parietal cortex</td>
<td>72</td>
<td>-2.139</td>
<td>3.243</td>
</tr>
<tr>
<td>Right dorsal thalamic nucleus</td>
<td>62</td>
<td>-3.957</td>
<td>2.200</td>
</tr>
<tr>
<td>Inter hemispheric prelimbic cortex</td>
<td>60</td>
<td>-3.738</td>
<td>0</td>
</tr>
<tr>
<td>Left auditory parietal cortex</td>
<td>59</td>
<td>-2.797</td>
<td>3.444</td>
</tr>
<tr>
<td><strong>BZD vs. KOA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inter hemispheric prelimbic cortex</td>
<td>109</td>
<td>-3.225</td>
<td>0</td>
</tr>
<tr>
<td>Left dorsal striatum</td>
<td>103</td>
<td>0</td>
<td>3.636</td>
</tr>
<tr>
<td>Inter hemispheric cingulate cortex 3</td>
<td>87</td>
<td>0</td>
<td>3.595</td>
</tr>
<tr>
<td>Left primary somatosensory cortex</td>
<td>68</td>
<td>-3.444</td>
<td>0</td>
</tr>
<tr>
<td>Right insular cortex</td>
<td>68</td>
<td>-3.704</td>
<td>0</td>
</tr>
<tr>
<td>Right primary somatosensory cortex</td>
<td>66</td>
<td>-2.983</td>
<td>0</td>
</tr>
<tr>
<td>Right auditory cortex</td>
<td>53</td>
<td>-2.068</td>
<td>3.457</td>
</tr>
<tr>
<td>Right primary secondary motor cortex</td>
<td>45</td>
<td>-2.858</td>
<td>3.246</td>
</tr>
<tr>
<td>Inter hemispheric hypothalamus 1</td>
<td>44</td>
<td>-2.370</td>
<td>2.812</td>
</tr>
<tr>
<td>Left auditory parietal cortex</td>
<td>39</td>
<td>0</td>
<td>3.091</td>
</tr>
</tbody>
</table>
Table 2
ALFF changes in brain regions induce by BZD in KOA rats.

<table>
<thead>
<tr>
<th>ROI</th>
<th>NZcount</th>
<th>Min_T</th>
<th>Max_T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham vs. KOA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right insular cortex</td>
<td>378</td>
<td>-4.230</td>
<td>0</td>
</tr>
<tr>
<td>Right primary somatosensory cortex</td>
<td>308</td>
<td>-4.403</td>
<td>0</td>
</tr>
<tr>
<td>Inter hemispheric prelimbic cortex</td>
<td>302</td>
<td>-4.655</td>
<td>0</td>
</tr>
<tr>
<td>Inter hemispheric hypothalamus2</td>
<td>268</td>
<td>0</td>
<td>4.419</td>
</tr>
<tr>
<td>Right mesencephalon (inferior colliculus)</td>
<td>246</td>
<td>-2.845</td>
<td>3.935</td>
</tr>
<tr>
<td>Left primary somatosensory cortex</td>
<td>224</td>
<td>-4.513</td>
<td>0</td>
</tr>
<tr>
<td>Inter hemispheric ventral thalamic</td>
<td>217</td>
<td>0</td>
<td>3.716</td>
</tr>
<tr>
<td>Inter hemispheric ventral tegmental</td>
<td>213</td>
<td>0</td>
<td>3.874</td>
</tr>
<tr>
<td>Inter hemispheric periaqueductal gray</td>
<td>199</td>
<td>0</td>
<td>3.9934</td>
</tr>
<tr>
<td>Right insular cortex 2</td>
<td>146</td>
<td>-3.402</td>
<td>0</td>
</tr>
<tr>
<td>BZD vs. KOA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inter hemispheric hypothalamus 2</td>
<td>275</td>
<td>0</td>
<td>3.431</td>
</tr>
<tr>
<td>Right mesencephalon (inferior colliculus)</td>
<td>183</td>
<td>-2.349</td>
<td>3.616</td>
</tr>
<tr>
<td>Right intermedial entorhinal cortex</td>
<td>98</td>
<td>0</td>
<td>3.522</td>
</tr>
<tr>
<td>Inter hemispheric ventral tegmental</td>
<td>91</td>
<td>0</td>
<td>3.346</td>
</tr>
<tr>
<td>Inter hemispheric hypothalamus 1</td>
<td>84</td>
<td>-3.354</td>
<td>2.730</td>
</tr>
<tr>
<td>Inter hemispheric interpeduncular nucleus</td>
<td>80</td>
<td>0</td>
<td>3.750</td>
</tr>
<tr>
<td>Left dorsal hippocampus</td>
<td>70</td>
<td>0</td>
<td>3.494</td>
</tr>
<tr>
<td>Right auditory cortex</td>
<td>63</td>
<td>0</td>
<td>3.698</td>
</tr>
<tr>
<td>Inter hemispheric retrosplenial granular</td>
<td>63</td>
<td>0</td>
<td>2.840</td>
</tr>
<tr>
<td>Right dorsal thalamic nucleus</td>
<td>60</td>
<td>0</td>
<td>3.664</td>
</tr>
<tr>
<td>Right insular cortex 2</td>
<td>31</td>
<td>0</td>
<td>3.800</td>
</tr>
</tbody>
</table>

Discussion

In this study, we performed a network pharmacology analysis and found that the therapeutic effect of BZD on KOA involves the regulation of neurotransmitters in the central and peripheral nervous systems to reduce pain. To verify the therapeutic effect of BZD on KOA, multidimensional evidence was obtained from histomorphology, behavioral tests, suspension chip analysis, and UPLC-MS/MS analysis, and we found that BZD not only alleviated the pain and inflammatory responses in KOA animals but also
affected the expression of serum neurotransmitters and regulated the level of pain-related neuropeptides. Subsequent fMRI studies showed that BZD treatment reversed changes in several brain regions associated with the KOA model. The above results suggest that the therapeutic effect of BZD is closely related to neuromodulation, which helps BZD to be further explored and developed as a novel analgesic drug.

**Therapeutic Effect Of Bzd On Koa Involves Neurotransmitters**

Network pharmacology analysis showed that BZD contained seven effective medicinal components and 36 predicted targets in the treatment of subchondral bone in KOA (Fig. 1A). KEGG signaling pathway analysis showed that there was one key neural signaling pathway, the serotonergic synaptic pathway, that plays a role related to BZD in KOA (Fig. 1B). 5-HT is involved in athletic ability, pain, mental activities, and other physiological functions. 5-HT is 95% distributed in the periphery, but it plays an important regulatory role in the central nervous system as a key neurotransmitter [26]. Serotonin regulates bone remodeling and mediates KOA pain. The diploma of its expression was once positively correlated with the severity of pain. The 5-HT/5-HT$_3$ receptor can cause neuronal excitation, and the 5-HT$_3$ receptor antagonist can reduce this neuronal response [27]. 5-HT is involved in the regulation of peripheral pain by activating related ion channels and releasing cytokines and factor receptors. Inhibiting the reuptake of serotonin in presynaptic neurons prevents chronic pain.

- **Verification of the therapeutic effects of BZD** in vivo

To understand the effect of BZD on neurotransmitter pain transmission, we conducted animal experiments. We found that in the KOA group, the cartilage layer was thinner, the chondrocyte arrangement was disordered, the blood vessels crossed the tidal line, the tidal line was blurred, and the trabecular bone was thinner. However, BZD treatment significantly reverted these changes but not completely to normal (Fig. 2A). Unbalanced mechanical stress leads to cartilage matrix destruction and subchondral bone resorption—known as a bone formation imbalance. The intervention of BZD decreased the expression of MMP-3, MMP-9 and MMP-13, and increased the level of Col-II, confirming that BZD can effectively inhibit the progressive lesions of KOA (Fig. 2B–G). Important biomarkers of bone metabolism, RANKL/OPG, ALP, and TRACP, were upregulated in KOA and downregulated with BZD treatment (Fig. 2H–K), suggesting that KOA promotes abnormal activities of osteoblasts and osteoclasts. Meanwhile, BZD may ameliorate this bone metabolic imbalance. Assessment of pain-related behavior showed that KOA decreased PWT and PWL, while BZD enhanced PWT and PWL (Fig. 2L & M), confirming the analgesic effect of BZD. These data demonstrate that surgery successfully established a rat model of KOA and that BZD alleviates KOA-induced morphological symptoms and bone metabolic manifestations. Importantly, we confirmed the analgesic impact of BZD in a KOA rat model.

Inflammatory cytokines are not only the initiator of KOA, but also the inducer of KOA development [28–30]. On the one hand, the manufacturing of inflammatory aspects stimulates the manufacturing of MMPs and promotes cartilage degeneration; on the other hand, it promotes osteoclast activation,
improves bone transformation, mediates bone remodeling, and reduces the neuroreceptor threshold, leading to peripheral sensitization. The expressions of IL-1α, IL-5, IL-7, IL-12 (P70), IL-17A, IL-18, M-CSF, G-CSF, RANTES, MIP-3α, MCP-1, and TNF-α in serum were upregulated in KOA group. The anti-inflammatory cytokines IL-13 and IL-4 decreased, and BZD reversed this situation (Fig. 3A–H and Fig. S4A–F). These data suggest that the inflammatory response and bone remodeling are enhanced in KOA and that BZD may mitigate these changes.

**Exploration Of The Potential Neural Mechanisms Underlying The Therapy Effects Of Bzd**

5-HT and its metabolites were drastically multiplied in the pain model [31], which is steady with the trend of the network pharmacological analysis and the UPLC-MS/MS serum detection. The binding of 5-HT and 5-HT₃ stimulates neurons to release neuromediators, such as SP and CGRP, which induce pain and mediate inflammation [32]. KOA pain was related with the expression levels of SP, CGRP, NPY, and TH. SP promotes inflammation via stimulating synovial fibroblasts to release inflammatory mediators [33–35]. CGRP is a key pro-inflammatory factor. In vitro studies have tested that CGRP can promote the proliferation of vascular endothelial cells and accordingly promote bone vascularization. The neuropeptides NPY and TH secreted by the sympathetic nerve participate in bone metabolism and promote inflammation. The expression of NPY is increased in inflammatory pain models, and exogenous NPY induces damage resistance in inflammatory and neuropathic pain models [36]. Changes in mechanical stress cause TH⁺ nerve fibers to develop in the subchondral bone. VEGFA promotes inflammation, macrophage recruitment, angiogenesis, and invasion of vegetative neurons into the bone-cartilage junction; additionally, it directly activates sensory neurons [37]. The adjustments of neuropeptide in this learn about had been steady with the consequences of preceding studies. Although BZD intervention can reverse these changes and relieves pain, reducing the inflammatory response via modulating neuropeptide expression may be the underlying mechanism of the effects of BZD.

The neuroregulatory mechanisms associated with BZD treatment prompted us to explore the impact of KOA surgery and BZD treatment on various brain regions. KOA can produce persistent chronic pain-related to nervous system function, and secondary changes in brain activity can be detected by imaging. By comparing the brain area changes of the three groups in this study, two findings were consistent with those of previous studies: 1) KOA leads to changes in ReHo and ALFF in the cortex, hippocampus, hypothalamus, midbrain, and diencephalon; and 2) The most significant changes were in the cortex, hypothalamus, and hippocampus were most significant (Fig. 5, Tables 1 and 2). In addition, the insular cortex showed the most significant changes. There were also corresponding changes in the striatum and cingulate gyrus. It has been established that the hippocampus and insula are closely associated with chronic pain in osteoarthritis [38]. In chronic osteoarthritis pain, the cingulate gyrus gray matter is reduced, and insular and nucleus accumbens functional connections are reduced [39]. Our experiment showed increased functional connections between the insula and the external thalamus, hippocampus, cortex, hypothalamus, and striatum (Fig. 6). Thus, KOA pain is subject to complex central nervous system
regulation. BZD plays an analgesic role through the functional connection between the cerebral cortex, thalamus, and hippocampus.

Conclusions

Through network pharmacology analysis, this study found that the therapeutic effect of BZD on KOA is inseparable from the regulation of neurotransmitters essential to the central and peripheral nervous system. Histomorphology, behavioral tests, suspension chip analysis, and UPLC-MS/MS evidence were used to verify the therapeutic effect of BZD on KOA. We found that BZD reduced the pain and inflammatory response in KOA animals, affected the levels of neurotransmitters in serum, and significantly impacted the expression of neuropeptides. Resting-state fMRI analysis showed that BZD treatment reversed changes in the cortical, thalamic, and hippocampal regions associated with the KOA model. These consequences show that the therapeutic impact of BZD is intently associated to the neural mechanisms that adjust pain. Our results are helpful to understand the brain functional connectivity between BZD and KOA, and provide further theoretical basis for better clinical application of BZD.

Declarations

Acknowledgements

We would like to give our sincere gratitude to the reviewers for their constructive comments.

Author Contributions

Conceptualization, X.T. and J.Z.P.; data curation, X.T. and Q.L.; formal analysis, L.L.W. and D.H.Z.; funding acquisition, X.H.L.; investigation, Z.S.Z. and Y.F.H.; methodology, Y.H.Z., M.M. and Z.P.Y.; writing original draft, X.T.; J.W. and Q.L.; writing—review & editing, X.H.L. All authors have read and agreed to the published version of the manuscript.

Funding

This study was supported by the National Natural Science Foundation of China (No. 81873319), Fujian Provincial Health and Health Commission Science and Technology Program Project TCM Clinical Research (No. 2021zylc52), and the Science and Technology Programs Pilot Project of Fujian Province (No. 2021Y0032).

Data availability statement

The datasets for this study can be found in the jianguoyun [https://www.jianguoyun.com/p/DYgBPvwQstXtChi2n-YEIAA].

Ethics statement
The animal study was reviewed and approved by the Animal Care and Use Committee of the Fujian University of TCM (Approval number: 2019062).

Consent to Participate

Not applicable.

Consent for Publication

All the authors have read the manuscript and agree for this publication.

Conflict of Interest

The authors declare no competing interests.

References


Figures
Figure 1

Predicted targets of BZD therapy for subchondral bone and neural signaling pathway of pain. Note: (A). Six databases: Gene Cards Database, OMIM Database, PharmGkb Database, Therapeutic Target Database, DrugBank Database and GEO Database combined analysis and intersection with BZD potential targets to obtain 36 predicted targets of BZD for subchondral bone of KOA. (B). KEGG enrichment
analysis showed that serotonergic synapse pathway was associated with pain of KOA. The binding of 5-HT to 5-HT₃ is the key to neuronal excitation.

Figure 2

Evidence of pathological function of KOA and pain sensitivity and therapeutic effect of BZD. Note: (A) Representative images of H&E, safranin O-fast green and Masson staining. Scale means 200 μm. Compared with the sham group (left column), the hyaline layer was thinner, chondrocytes were disordered, tidal lines were blurred and discontinuous, trabecular bone was thinner, the structure was sparse and new bone appeared in the model group (middle column). These changes were reversed by BZD (right column). (B) and (C) are the representative image of the protein expression levels of Col-II, MMP-3, MMP-9, MMP-13 in cartilage. (D) Quantitative analysis of Col-II relative protein expression. KOA significantly decreased Col-II protein level, while BZD significantly increased Col-II protein level. (E-G) Quantitative analysis of the protein levels of inflammatory related protein (E) MMP-3, (F) MMP-9, and (G) MMP-13 in cartilage. (H) is the representative image of bone homeostasis protein. (I-K) Quantitative analysis of (I) RANKL/OPG, (J) ALP, and (K) TRACP in subchondral bone. All the results have the same
trend. KOA significantly increased protein levels, while BZD significantly decreased the increased protein levels induced by KOA. (L) PWT and (M) PWL are used to assess pain. KOA significantly reduced the PWT and PWL, whereas BZD treatment significantly increased PWT and PML of animals. P < 0.05, P < 0.01, Sham vs. KOA; *P < 0.05, **P < 0.01, KOA + BZD vs. KOA.

Figure 3

BZD regulates the contents of inflammatory factors and neurotransmitters in serum. Note: In the serum inflammatory factors (A-H), Quantitative analysis of (A) IL-1α, (B) IL-5, (C) IL-12, (D) IL-17A, (E) RANTES, (F) TNF-α, (G) M-CSF and (H) IL-13 in serum. KOA significantly increased the contents of IL-1α, IL-5, IL-12, IL-17A, RANTES, TNF-α and M-CSF. Whereas BZD significantly inhibited their contents in serum. KOA significantly decreased the content of IL-13, whereas BZD significantly increased the decreased content induced by KOA. In the serum neurotransmitter (I-R), Quantitative analysis of (I) GABA, (J) E, (K) GSH, (L)
Kyn, (M) Met, (N) VMA, (O) TrpA, (P) TyrA, (Q) Spd and (R) BALa in serum. KOA significantly decreased the contents of GABA, E, GSH, Kyn, Met and VMA. Whereas BZD significantly increased their contents in serum. KOA significantly increased the contents of TrpA, TyrA, Spd and BALa. Whereas BZD significantly decreased the increased contents induced by KOA. P < 0.05, P < 0.01, Sham vs. KOA; *P < 0.05, **P < 0.01, KOA + BZD vs. KOA.

Figure 4

BZD relieves pain by regulating neuropeptides. Note: (A–D) are the serum contents of (A) SP, (B) CGRP, (C) NPY and (D) TH. KOA significantly increased the contents of SP, CGRP, NPY and TH in serum. Whereas BZD significantly reduced the elevation of serum SP and TH. CGRP and NPY showed the trend but did not reach a significant level. (E–G) Representative images of the protein expression levels of SP, CGRP, NPY, TH, VEGFA and 5-HT₃ in subchondral bone. (H–M) Quantitative analysis of the protein levels of neuropeptide related proteins. KOA significantly increased the protein levels of (H) SP, (I) CGRP, (J) NPY, (K) TH, (L) VEGFA and (M) 5-HT₃ in subchondral bone, Whereas BZD significantly inhibited the increased level induced by KOA. P < 0.05, P < 0.01, Sham vs. KOA; *P < 0.05, **P < 0.01, KOA + BZD vs. KOA.
Figure 5

Representative resting-state fMRI images. Note: (A). changes of ReHo induced by KOA treatment. SPM analysis of Sham-KOA treatment (model). (B). changes of ReHo induced by BZD treatment. SPM analysis of BZD treatment-KOA treatment (model). The yellow areas represent brain regions with enhanced ReHo; The blue areas represent areas where ReHo values have decreased. (C). changes of ALFF induced by KOA treated. SPM analysis of Sham-KOA treatment (model). (D). changes of ALFF induced by BZD treatment. SPM analysis of BZD treatment-KOA treatment (model). The yellow areas represent brain regions with enhanced ALFF; The blue area represents the area where the ALFF value decreases.
Figure 6

Comparison of the functional connectivity (FC) of the brain after KOA induction and BZD treatment. Note: Left: Significant differences of the correlation in the average FC between SHAM and KOA1; Right: Significant differences of the correlation in the average FC between BZD and KOA1; Black point: Decreased FC values; white point: Increased FC values.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementalmaterial.docx