

A Water Extract of Benefiting-Bone Capsule Improves Osteoporosis *via* Transcriptional and Translational Regulation of Key Factors in Notch and Wnt/ β -catenin Signaling Pathways in Ovariectomized Rats

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Abstract

Benefiting-Bone Capsule (BBC) is a Chinese herbals formula that shows promise in the treatment and prevention of osteoporosis (OP), but the mechanisms underlying its anti-osteoporotic effects are yet to be established. Here we detect the influence of the extract on several targets of the Notch and Wnt/ β -catenin signaling pathways in bone tissue of ovariectomized rats as a model of OP with the aid of real-time PCR, western blot, and immunohistochemical staining. The water extract of BBC exerted a pronounced beneficial effect on OP rats and activated the Wnt/ β -catenin pathway through regulating Wnt3a, Wnt10b protein, and glycogen synthesis kinase 3 β (GSK-3 β) mRNA. Furthermore, the BBC extract inhibited Jagged1, 2 protein and Notch 1,2 mRNA of the Notch signaling pathway. We detected simultaneous changes in the levels of a number of downstream transcripts, including increased runt-related transcription factor 2 (Runx 2) and decreased peroxisome proliferator-activated receptor γ (PPAR γ), which were closely correlated with the treatment effect. Based on the collective findings, we propose that the water extract of BBC regulates OP-associated factors through modulation of key transcripts and proteins in the Notch and Wnt/ β -catenin pathways. Our results provide further mechanistic evidence supporting the clinical efficacy of BBC in the treatment of OP.

Keywords: Water extract of benefiting-bone capsule; Osteoporosis; Notch; Wnt/ β -catenin

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Introduction

Osteoporosis (OP) is a metabolic bone disease, characterized by low bone mass and microarchitectural deterioration, resulting in increased bone fragility and susceptibility to fracture [1]. Typically, OP patients have a high rate of bone remodeling with an imbalance leading to bone resorption in excess of bone formation. Globally, osteoporosis affects approximately one-tenth of women by 60 years of age and two-fifths of women by 80 years of age [2]. In view of the increasingly aging population, osteoporosis is becoming a global health problem and its incidence and consequent economic and social costs are expected to rise in the future [3].

Various drugs have been developed to reduce osteoporosis and risk of associated fractures, such as calcium, vitamin D, and bone resorption inhibitors (estrogen, selective estrogen receptor modulators, bisphosphonates). However, these drugs

also induce several side effects, and the long-term curative effects of specific agents (such as fluoride) are uncertain [4]. Since Youyou Tu was awarded the Nobel Prize in 2016, Traditional Chinese Medicine (TCM) has become a considerable focus of research in the quest for to developing safer and more effective anti-osteoporotic drugs.

Benefiting-Bone Capsule (BBC) is an herbals formula, mainly consisting of seven traditional Chinese medicines (TCM) including Epimedium, Chinese wolfberry, and Radix Rehmanniae Preparata. A BBC drug serum has been shown to promote proliferation, differentiation, and mineralization of OB [5] and apoptosis of OC [6], *in vitro*, while *in vivo*, a water extract of BBC ameliorated osteoporosis in ovariectomized rats [7]. Clinically, the water extract of BBC improved symptoms of OP with no obvious toxicity or adverse reaction revealed during the six-month of experimental period [8], supporting its potential efficacy in the treatment and prevention of OP.

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To explore the mechanisms underlying the anti-osteoporotic effects of the water extract of BBC, we focused on the Wnt/ β -catenin and Notch signaling pathways. The Wnt protein family includes a number of cysteine-rich glycoproteins [9], including two ligands that are positively correlated with bone remodelling, Wnt3a [10-12] and Wnt10b [13,14]. In theory, the canonical Wnt/ β -catenin signaling pathway is activated by Wnt proteins through release and accumulation of intracellular β -catenin, which are degraded by the proteasome through the key enzyme, glycogen synthesis kinase 3 β (GSK-3 β) when the pathway is turned off [15,16]. Increasing the Wnt signal suppresses Notch signaling through the combined activities of GSK-3 β and Notch 2 [17]. Conversely, increasing Notch signaling leads to inhibition of the Wnt downstream protein, β -catenin [18]. The Wnt and Notch signaling pathways co-regulate several downstream targets, including the adipogenic differentiation factors, peroxisome proliferator-activated receptor γ (PPAR γ).

We propose that the water extract of BBC exerts anti-osteoporotic effects via regulation of the Notch and Wnt/ β -catenin signaling pathways. Here, we focused on the effects of BBC extract on several key targets of these two signaling pathways in bone tissue. Our findings provide a basis for further exploration of BBC as an anti-osteoporotic drug and development of TCM as an effective treatment option in this field.

Materials and Methods

Materials

Animals: Specific Pathogen-Free (SPF) female Sprague-Dawley (SD) rats (aged 3 months and weighing 250 ± 20 g) were provided by Guangdong Medical Laboratory Animal Center (Guangzhou, China) (Certificate Number: Guangdong SCXK 2013-0002).

Experimental medications: The liquid extract of benefiting-bone capsule (Batch number: 20150601) containing 2.94 g active ingredient per gram was purchased from Guangzhou Boji Medical Biotechnological Co. Ltd. (Guangzhou, China), liquid extract has 2.94 g active ingredient per gram. The positive control drug, Fosamax (Product number: 130124), was purchased from MSD Pharmaceutical Co. Ltd. (Hangzhou, China).

Reagents: The alkaline phosphatase assay kit was acquired from Roche Ltd. (Basel, Switzerland) and the AST activity assay kit from Sekisui Medical Co., LTD. (Tongren Japan). OPG, BGP, and E2 ELISA test kits were purchased from Cloud-Clone Corp. (Wuhan China). Rat N1ICD and Jagged-1 polyclonal antibodies were obtained from Abcam plc. (Cambridge UK). Rat β -catenin, Wnt3a and GAPDH polyclonal antibodies were purchased from Cell Signaling Technology, Inc. (Boston Massachusetts USA). And rat Jagged-2 polyclonal antibody from Merck Millipore Co. (Darmstadt, Germany). Rat Wnt10b polyclonal and goat anti-rabbit secondary antibodies were obtained from OriGene

Technologies, Inc. (Maryland USA) and EarthOx (Wuhan, China) respectively. Bovine serum albumin was purchased from Roche Ltd. (Basel, Switzerland). RNA reverse transcription and RT-PCR SYBR[®] kits were from TaKaRa Biotechnology (Dalian) Co., Ltd. (Dalian, China) Phenyl methane sulfonyl fluoride (PMFS) were purchased from Sigma-Aldrich Co. Ltd. (Shanghai, China) and the bicinchoninic acid (BCA) protein assay kit from KeyGEN Biotech. Co. Ltd. (Nanjing, China). SDS-PAGE gel preparation kit, protein loading buffer (5x) and electrophoresis liquid were acquired from the Beyotime Institute of Biotechnology (Haimen, China). Other commercially available reagents and chemicals used were of analytical grade.

Instrumentation: The following equipment was employed: a dual X-ray absorptiometer (Lunar Prodigy, GE Co., Pittsburgh, PA, USA), a microplate absorbance reader (Model 680, Bio-Rad Co., Hercules, CA, USA), micro-CT system (u-CT80, SCANCO Medical AG, Switzerland), multi-function biomechanics tester (MTS Model 858, Bionix Co., Toledo, OH, USA), inverted phase-contrast microscope (Model CKX41, Olympus Co., Tokyo, Japan), fluorescence spectrophotometer (Nano Drop 1000, Thermo Co., Waltham, MA, USA), G-Storm Gradient PCR thermal cycler (Veriti 96-Well, Applied Biosystems Co.), quantitative fluorescence PCR system (Light Cycler[®] 480, Roche Diagnostics Company Ltd.), gel imaging system (Bio-Rad Co.) and biomechanical instrumentation (3510-AT, BOSE Co., USA).

Methods

Components analysis of BBC liquid extract: Qualitative and quantitative analyses of the chemical constituents of BBC liquid extract were performed by using HPLC-Q/TOF-MS. The Agilent 6200 Series 6210 G1969A TOF LC/MS Mass Spectrometer system (Waldbronn, Germany) was employed for data acquisition and Mass Hunter Qualitative Analysis DA Software B.06.00 (Santa Clara, CA, USA) for data analysis. Errors were calculated by analysing three batches of BBC liquid extract. All reference standards of BBC liquid extract (>98% purity) were purchased from the Guangdong Institute for Drug Control (Guangzhou, China). Chromatographic separation was performed on COSMOSIL 38020-41 5C18-MS- (4.6 \times 250 mm) (Nacalai Tesque, Inc., Japan) at 25 $^{\circ}$ C. The mobile phase comprised a mixture of water containing 100% formic acid (A) and methanol (B). Linear gradient elution was conducted as follows: 0–45 min at 10–100% B, 45–60 min at 100% B. The flow rate was 1.0 mL/min and injection volume was 25 μ L. UV detection was performed at 210 nm.

Animals and groups: SPF rats were reared by the Jinan University Medical Laboratory Animal Center and had free access to water and standard laboratory chow (1.01% Ca²⁺, 0.78% P³⁺).

Eighty-four rats were randomly divided into an ovariectomized non-treatment group (OVX-NT) (70 animals) that developed OP and sham-operated group (14 animals). After 20 weeks, rats were subjected to the dual-energy X-ray bone mineral

density (BMD) test. The OVX model was successfully established as described previously [19,20]. The OVX-NT group was randomly divided into five groups of 14 rats: non-treatment group (OVX-NT), a low-dose BBC group (BBC-L), medium-dose BBC group (BBC-M), high-dose BBC group (BBC-H) and a Fosamax-treated positive control group (FOS). Rats underwent treatment for 12 weeks.

BBC dosage was as follows: 0.52 g/200 g^{*}d (low-dose group), 1.56 g/200 g^{*}d (middle-dose group), and 4.68 g/200 g^{*}d (high-dose group). BBC and Fosamax were dissolved in distilled water to unify the volume and administered via oral gavage. The dosage of gavage administration in rats was 1, 3 and 9 times of the normal dose used in humans, as measured based on the active ingredient. Treatment regimens were as follows (Table 1): (1) sham-operated, water (Sham group); (2) OVX, water (OVX-NT group); (3) OVX, 0.52 g/200 g/day (low-dose BBC-L group); (4) OVX, 1.56 g/200 g/day (middle-dose BBC-M group); (5) OVX, 4.68 g/200 g/day (high-dose BBC-H group); and (6) OVX, 0.514 mg/kg/day Fosamax (FOS group).

Table 1: Treatment groups (dose measurements based on the active ingredient)

Group	Model	Drug	Dose	Dosing period	Administration
Sham group	Sham	Water	/	12weeks	Oral gavage
OVX-NT group	OVX	Water	/	12 weeks	Oral gavage
BBC-L group	OVX	BBC	0.52 g/200 g/day	12 weeks	Oral gavage
BBC-M group	OVX	BBC	1.56 g/200 g/day	12 weeks	Oral gavage
BBC-H group	OVX	BBC	4.68 g/200 g/day	12 weeks	Oral gavage
FOS group	OVX	Fosamax	0.514 mg/kg/day	12 weeks	Oral gavage

Dual-energy X-ray absorptiometry (BMD): After 20 weeks, BMD (bone mineral density) was evaluated via dual X-ray scanning. Rats were anesthetized and scanned. Bone mineral densities of femora and fourth and fifth lumbar vertebrae (LV4, 5) were measured.

Enzyme-Linked Immunosorbent Assay (ELISA): Blood plasma was collected using the abdominal aortic method. A serum separator tube was used and samples allowed to clot for 30 min before centrifugation for 10 min at 3000×g. Serum was removed and assayed immediately or divided into aliquots and stored at -20 or -80. Repeated freeze-thaw cycles were avoided. OPG, BGP, and E2 ELISA kits were used to test the respective indexes. According to protocol instructions, the microtiter plate reader was used as the standard for determination of test results.

Assessment of biochemical parameters: Serum was obtained with the above method. Serum levels of Ca²⁺, P³⁺, ALP

[Alkaline Phosphatase] and AST [Aspartate Aminotransferase] were detected using an automatic biochemistry analyzer.

Micro-CT: Right femora and LV 5 were separated, dissected free of soft tissues and stored at -80°C for subsequent analyses. The microarchitecture of trabecular bone in the right proximal femora and LV4 was analyzed using micro-CT (60 KV, 50 W). The same specimen was scanned to obtain images of different sections and distal femoral stem epiphyseal and vertebral scans performed in three spatial dimensions. Micro View software was employed to calculate: trabecular thickness (Tb. Th), trabecular number (Tb. N), trabecular separation (Tb. Sp) and Trabecular Bone Pattern Factor (TBPF).

Three-point bending and compression tests: Samples were obtained using the method described above. The maximum impact force of different groups in the three-point bending test was investigated using rat femoral samples. The maximum axial compressive load of different groups was in a compression test using rat LV 5.

Hematoxylin-Eosin (HE) staining: Bone femoral tissues were decalcified and sliced with paraffin embedding, followed by processing with HE staining. Sections were examined and images obtained under an optical microscope.

Immuno histochemical staining: One third of the right distal femur was fixed with 4% paraformaldehyde, followed by fixing with 20% EDTA for decalcification at 4°C, dehydrated, and embedded in paraffin. Five micron paraffin sections were prepared for immunohistochemical staining. Slices were dewaxed using xylene and hydrated with an alcohol gradient. Endogenous peroxidase activity was quenched (3% hydrogen peroxide) and the slices incubated with Jagged1, Wnt3a and Wnt10b primary antibodies respectively (1:1000 dilution), at 37°C for 1h. Slices were washed three times with phosphate buffer, incubated with streptavidin- HRP- conjugated secondary antibodies at 37°C for 10 min, and washed another three times with phosphate buffer. Diamino benzidine (DAB) - colored slices were then stained with hematoxylin and dehydrated using alcohol. Xylene was added and the slices sealed using neutral gum.

Quantitative fluorescence PCR: Chopped rat femora were treated with liquid nitrogen and ground into powder. Total RNA was extracted from femora by triturating several times with TRIzol reagent and allowing to stand at room temperature for 5 min. Chloroform was added (1/5th-volume of TRIzol) and the sample blended in a vortex mixer. After standing at room temperature for 5 min, the mixture was centrifuged (12,000 g) for 5 min at 4°C. The top 70% of the aqueous phase (0.5 mL) was transferred to an Eppendorf tube and shaken with isopropyl alcohol (0.25 mL), 0.8 M aqueous sodium citrate solution (0.125 mL) and 0.125 M aqueous NaCl solution (0.8 mL). After standing at room temperature for 10 min, the mixture was centrifuged (12,000 g) for 15 min at 4°C, washed twice with 75% ethanol and the sediment dissolved in Di Ethyl Pyro Carbonate (DEPC)-treated water (20 µL). Avoiding bubbles, RNA samples (1 µL) were placed in the spectrophotometer and the ratio of absorbance at 260 nm and

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280 nm (A260/A280) determined to provide an assessment of purity. Total RNA (1 μ g) was reverse-transcribed into cDNA. Template DNA was used in gene-specific PCR for PPAR γ , Runx 2, Notch 1, Notch 2, GSK-3 β , β -Catenin and GAPDH mRNA. Details of the primers are listed in Table 2. Quantitative PCR for gene expression was performed in 96-well plates in a total reaction volume of 20 μ L per well, comprising 2 \times SYBR green master mix diluted gene primers (10 μ L), cDNA (2 μ L), forward primer (0.8 μ L) or reverse (0.8 μ L), primer and DEPC-treated water (6.4 μ L). Quantitative analysis was performed using a Roche Light Cycler 480 Sequence Detection System. Operating conditions were as follows: 95°C for 30 s, 95°C for 5 s, and 60°C for 30 s for a total of 40 cycles. The fluorescence signal was collected at the end of the second step of each cycle. Each sample was analyzed in triplicate and average Ct values calculated. Gene expression was quantified using the $\Delta\Delta$ Ct approach.

Table 2: Primer sequences for quantitative fluorescence PCR

Gene name	Primer sequence
PPAR γ	Forward: 5'-GACCACTCCCATTCCTTTGACAT -3' Reverse: 5'-TCAGAATAATAAGGCGGGGACG -3'
Runx 2	Forward: 5'-AGCGGACGAGGCAACAGTTT-3' Reverse: 5'-CCTAAATCACTGAGGCGGTTCAG-3'
Notch 1	Forward: 5'-CCAGGGTGGTCAGGAAAGTC -3' Reverse: 5'-GCAGCCACAGATGTATGAAGACTC -3'
Notch 2	Forward: 5'-AGTGGTATGGACTGTGAGGAGG -3' Reverse: 5'-CAGGAGAAGGTGTTCACTTTGTC -3'
GSK-3 β	Forward: 5'-ACACCTGCCCTCTTCAACTTTACC-3' Reverse: 5'-ATTGGTCTGTCCACGGTCTCCA-3'
β -Catenin	Forward: 5'-ACTCTAGTGCAGCTTCTGGTTCTG-3' Reverse: 5'-CTCGGTAATGTCTCCCTGTC -3'
GAPDH	Forward: 5'-CAACGGGAAACCCATCACCA-3' Reverse: 5'-ACGCCAGTAGACTCCACGACAT-3'

Western blot analysis: Rat femora were subjected to cell lysis to extract proteins. Total protein concentrations were determined using the BCA protein assay kit. Proteins (30 μ g) were separated via 12% SDS-PAGE and transferred onto Poly vinylidene difluoride (PVDF) membranes. Membranes were blocked with buffer containing 0.05% Tween-20 and 5% skimmed milk and reacted sequentially with the appropriate primary and secondary antibodies. The primary antibodies used were specific for Jagged 2, β -Catenin and GAPDH (1:1000 dilutions) and secondary antibody was membranes were washed and rinsed with Enhanced Chemiluminescence (ECL) detection reagents, and immunoreactive bands visualized using ECL substrates and an X-ray film processor. Protein expression was measured using Quantity One[®] software.

Statistical analysis

Values are expressed as means \pm standard deviations (SD). The significance of differences between the two experimental

groups was estimated via one-way analysis of variance. Data were considered statistically significant at p values < 0.05. All statistical evaluations were performed using SPSS version 19.0.

Results

Chemical constituents of BBC liquid extract

Qualitative and quantitative analyses of the chemical constituents of the liquid extract were performed using HPLC-MS. Five bioactive components were identified, as shown in (Figure 1).

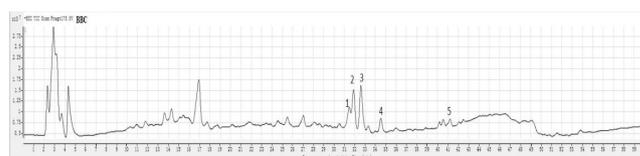


Figure 1: Total ion chromatography of BBC liquid extract via HPLC-MS. (1) Epimedin A, (2) Epimedin B, (3) Icarin, (4) Epimedin C, (5) Baohuside I

Establishment of the OP model and effects of the extract on BMD

At 20 weeks after OVX, BMD of LV4, 5, right femur and left femur was significantly reduced in the OVX-NT group relative to the sham-operated group (p < 0.05), indicating the successful establishment of the OP model (Figure 2).

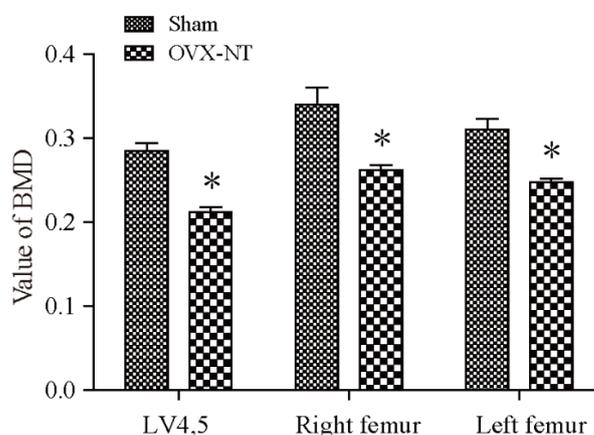


Figure 2: Compared with the sham-operated group, BMD of LV4, 5, right femur and left femur decreased significantly in the OVX-NT group, indicating successful establishment of the OP model. *p < 0.05 indicates OVX-NT group vs Sham group.

After the treatment period, BMD of the OVX-NT group was significantly lower than that of the Sham group reduction (p < 0.05). Compared with the OVX-NT group, BMD of the parameters examined in the FOS and BBC groups was significantly increased (p < 0.05); In particular, highest BMD was observed for the BBC-H. The results are shown in (Figure 3).

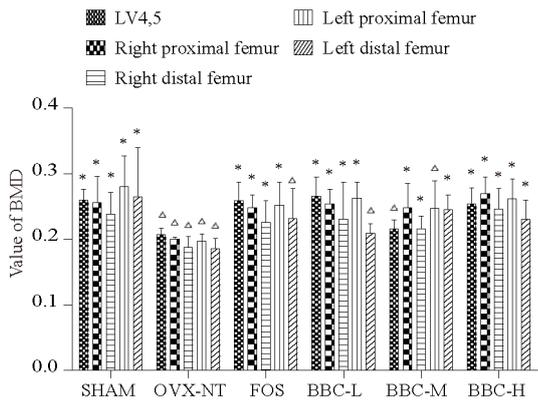


Figure 3: After 12 weeks of treatment, mice were anesthetized and the whole body scans conducted. BMD values of the whole body, femora, tibiae, and LV4, 5 were measured. BMD in proximal and distal femurs was significantly lower in the OVX-NT than Sham group. Compared with the OVX-NT group, BMD of the lumbar spine, left and right proximal femora and right distal femur in the FOS group was increased. BMD values in the BBC treatment groups were significantly increased. $\Delta p < 0.05$ indicates vs sham group, * $p < 0.05$ indicates vs OVX-NT group.

The water extract of BBC improves E2, BGP and OPG in serum

Compared with the sham group, Estradiol (E2), Bone Gla Protein (BGP) and Osteoprotegerin (OPG) levels in sera of OVX-NT groups were significantly lower ($p < 0.05$). Notably, serum E2, BGP and OPG levels of FOS, BBC-H, and BBC-M groups were significantly increased relative to those of the OVX-NT group ($p < 0.05$). We additionally observed increased serum E2 and OPG levels in BBC-L rats increased ($p < 0.05$). The results are presented shown in (Figures 4-6).

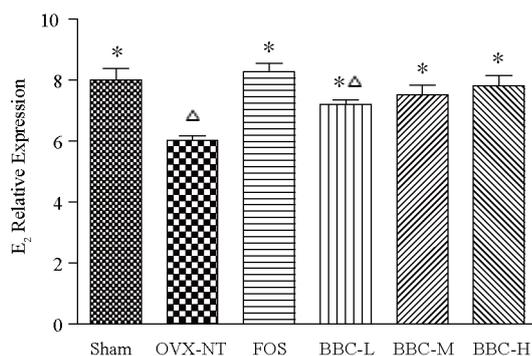


Figure 4: E2 levels in the six groups. Compared with the Sham group, E2 levels in serum samples of the OVX-NT group was significantly lower ($\Delta p < 0.05$). Compared with the OVX-NT group, E2 levels in FOS, BBC-L, BBC-M, and BBC-H groups were markedly increased (* $p < 0.05$). $\Delta p < 0.05$ indicates vs. Sham group * $p < 0.05$ indicates vs. OVX-NT group.

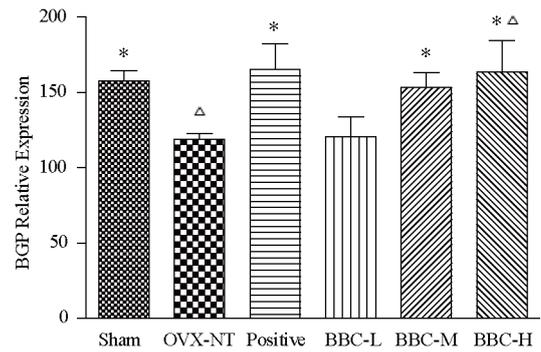


Figure 5: BGP levels in the six groups. Compared with the Sham group, BGP levels in serum samples of the OVX-NT group were significantly lower ($\Delta p < 0.05$). Compared with the OVX-NT group, BGP levels of positive control, BBC-M, and BBC-H groups were significantly increased. $\Delta p < 0.05$ vs Sham group * $p < 0.05$ vs. OVX-NT group.

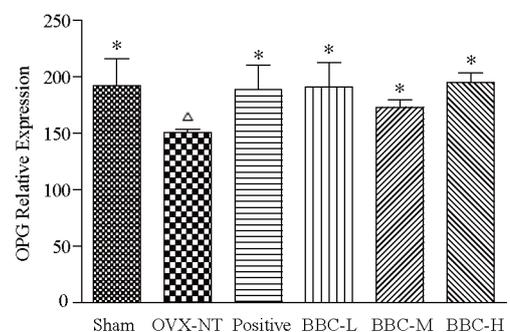


Figure 6: OPG levels in the six groups. Compared with the Sham group, OPG levels in serum samples the OVX-NT group was significantly lower. Compared with the OVX-NT group, OPG levels of FOS, BBC-L, BBC-H and BBC-M groups were significantly increased. $\Delta p < 0.05$ vs Sham group * $p < 0.05$ vs OVX-NT group.

The water extract of BBC improves trabecular bone indicators

Micro-CT analysis revealed that compared with the Sham group, bone trabecular number (Tb. N) and trabecular thickness (Tb. Th) of the OVX-NT group were significantly lower ($p < 0.05$), while trabecular separation/spacing (Tb. Sp) and trabecular bone pattern factor (Tb. Pf) were markedly increased ($p < 0.05$). All treatment groups displayed similar trends in trabecular bone parameters Sham group (Figures 7 and 8).

HE staining of pathological sections of distal femur showed that trabecular bone of OVX-NT rats was reduced, sparse, irregular and partially fractured, compared with the Sham group. Poor connectivity between the trabecular bones contains a lot of trabecular bone blind side. The number of hematopoietic cells decreased along with an increase in marrow cavity and fat droplets. However, morphological structures in the BBC-H group were similar to those of the Sham group. Staining data are shown in (Figure 9).

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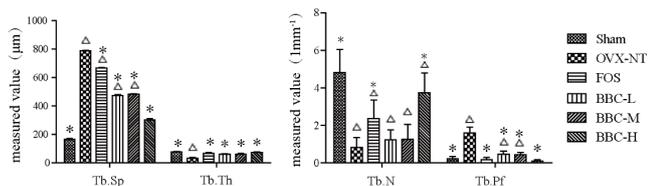


Figure 7: After 12 weeks of treatment, femur tissues were examined using Micro-CT. Results are expressed as means \pm standard deviations ($n=10$). Tb. N and Tb. Th values were significantly decreased in the OVX-NT, relative to the Sham group ($p<0.05$), while Tb. Sp and Tb. Pf values were significantly increased ($p<0.05$). Tb. N and Tb. Th values of FOS and BBC-H groups were significantly higher compared to the OVX-NT group ($*p<0.05$). Tb. N: Trabecular Number; Tb. Th: Trabecular Thickness; Tb. Sp: Trabecular Separation.

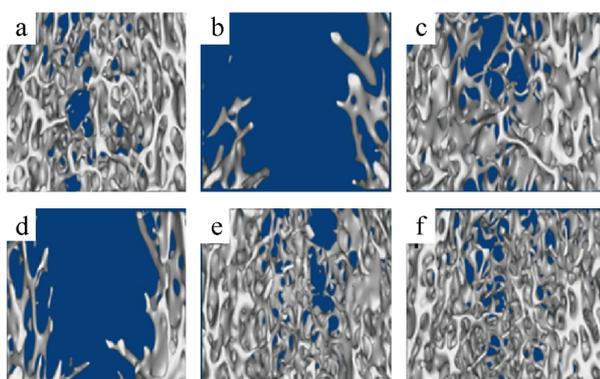


Figure 8: Micro-computed tomography analysis for (a) Sham, (b) OVX-NT, (c) FOS, (d) L-BBC, (e) M-BBC and (f) H-BBC groups. Compared with the Sham group, bone trabecula was loose and fractured and exhibited a loss in the OVX-NT group. Following oral administration of the BBC extract bone trabecular number was clearly increased and the trabecula more densely concatenated than that in the OVX-NT group. Tb. N: Trabecular Number; Tb. Th: Trabecular Thickness; Tb. Sp: Trabecular Separation; OVX-NT, ovariectomy and oral administration of normal saline; BBC, benefiting-bone capsule; Sham, sham-operated; FOS, treated with alendronate sodium; LBBC, Low-dose BBC group (0.71 g/kg•d); M-BBC, Middle-dose BBC group (2.13 g/kg•d); H-BBC, High-dose BBC group (6.39 g/kg•d).

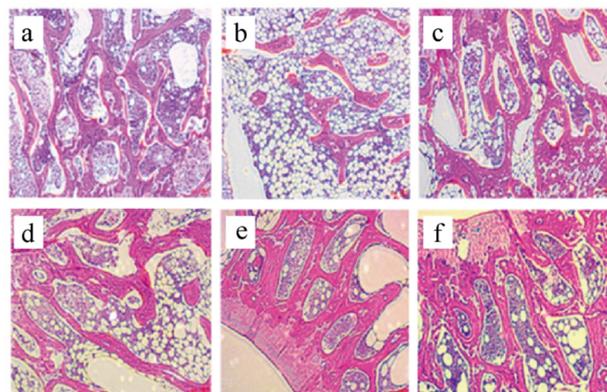


Figure 9: Histopathology of femurs from the six groups: (a) Sham, (b) OVX-NT, (c) FOS, (d) L-BBC, (e) M-BBC and (f) H-BBC groups. Hematoxylin and eosin staining (magnification, $\times 40$). OVX, ovariectomy; BBC, benefitingbone capsule; Sham, sham-operated; FOS, treated with alendronate sodium; L-BBC, Low-dose BBC group (0.71 g/kg•d); MBBC, Middle-dose BBC group (2.13 g/kg•d); HBBC, High-dose BBC group (6.39 g/kg•d). $\Delta p<0.05$ vs. Sham group $*p<0.05$ vs. OVX-NT group.

After 12 weeks of treatment, lumbar vertebral tissue was examined via micro-CT. Results are expressed as means \pm standard deviations ($n=10$). Tb. N and Tb. Th values were significantly decreased, while Tb. Sp and Tb. Pf values of the OVX-NT group were significantly increased, relative to the Sham group ($\Delta p<0.05$). Among the treatment groups, Tb. N and Tb. Th values of FOS and BBC-H groups were significantly higher compared with the OVX-NT group ($*p<0.05$), (Figure 10).

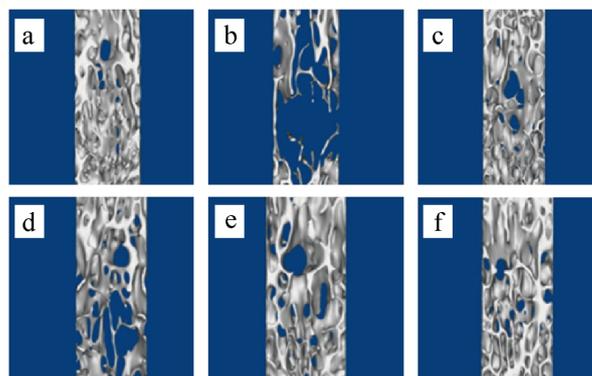


Figure 10: Micro-computed tomography analysis of LV5 for (a) Sham, (b) OVX-NT, (c) FOS, (d) L-BBC, (e) M-BBC and (f) H-BBC groups. Compared with the Sham group, the OVX-NT group exhibited loss of bone trabecula as well as loose and fractured appearance. Following oral administration of BBC, bone trabecula number was clearly increased and more densely concatenated relative to the OVX-NT group, with the HBBC group showing the greatest improvement. FOS, treatment with alendronate sodium; L-BBC, Low-dose BBC group (0.71 g/kg•d); M-BBC, Middle-dose BBC group (2.13 g/kg•d); H-BBC, High-dose BBC group (6.39 g/kg•d).

The water extract of BBC improves bone maximum load

Three-point bending and compression tests disclosed that relative to the Sham group, maximum load of LV5 and femur in OVX-NT rats was significantly lower ($p < 0.05$). Compared with the OVX-NT group, the maximum load of the femur was significantly increased in the FOS and BBC treatment groups ($p < 0.05$), while that of LV5 showed a marked increase in the BBC-H group ($p < 0.05$), (Figure 11).

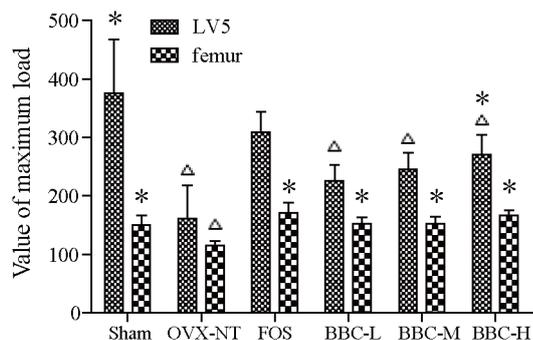


Figure 11: Compression tests to assess maximum load of LV5 and femur. FOS, group treated with alendronate sodium; BBC-L, Low-dose BBC group (0.71 g/kg•d); BBC-M, Middle-dose BBC group (2.13 g/kg•d); BBC-H, High-dose BBC group (6.39 g/kg•d). $\Delta p < 0.05$ vs. Sham group * $p < 0.05$ vs. OVX-NT group.

The water extract of BBC regulates key proteins and mRNAs of the Notch signaling pathway

The BBC extract inhibits Jagged1 protein expression: Immunohistochemical analysis Jagged1 distribution in the

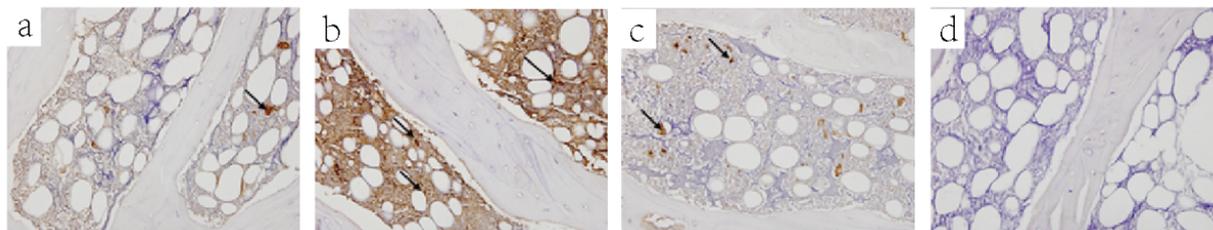


Figure 12: Expression of Jagged1 protein in the marrow cavity. Original magnification 400 \times . a, Sham group; b, OVX-NT group; c, BBC-H group (6.39 g/kg•d); d, negative control group.

bone marrow cavity on fat cell membranes. Compared with the OVX-NT group, expression of Jagged1 protein was lower in the sham-operated and BBC-H groups (Figure 12). The average density was determined using Image-Pro Plus 6.0 Image analysis software. In relation to the sham-operated group, expression of Jagged1 was significantly increased in the OVX-NT group ($p < 0.01$). The Jagged1 protein level was decreased in the BBC-H group, compared with the OVX-NT group (Figure 13).

The water extract of BBC enhances Runx2 mRNA and suppresses PPAR γ Notch1 and Notch2 mRNA: Q-PCR experiments showed that compared with the Sham group, expression of PPAR γ Notch1 and Notch2 mRNA levels were increased ($p < 0.05$), while that of Runx2 was decreased in the OVX-NT group ($p < 0.05$). In the BBC-H group, expression of PPAR γ , Notch1 and Notch2 mRNA was decreased ($p < 0.05$), to a significant extent, relative to the OVX-NT group (Figure 14).

BBC inhibits Jagged2 protein expression: Western blot data showed that relative to Sham group, expression of Jagged2 was significantly increased in the OVX-NT group ($p < 0.05$). Following treatment, we observed a marked decrease in Jagged2 protein expression ($p < 0.05$) in the BBC-H group, compared with the OVX-NT group (Figure 15).

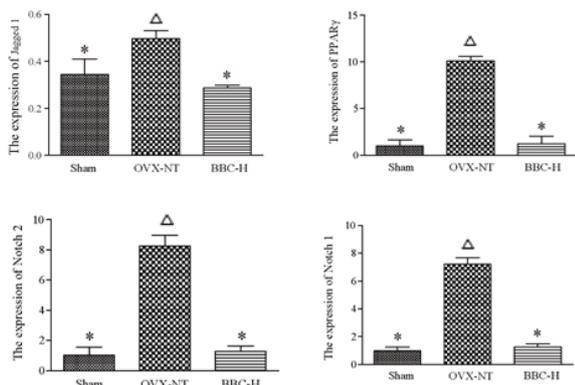


Figure 13: Relative expression quantity of *Jagged1* in the marrow cavity. Expression in the OVX-NT group was significantly increased compared with the Sham group. Notably, protein expression was significantly decreased after 12 weeks of treatment in the high-dose BBC-H, (6.39 g/kg·d). $\Delta p < 0.05$ vs Sham group, $*p < 0.05$ vs OVX-NT group.

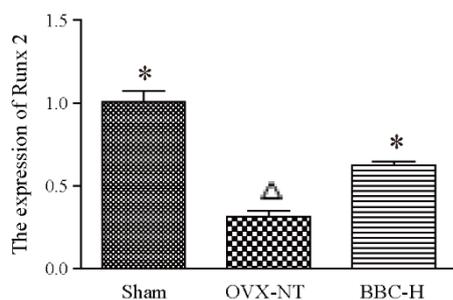


Figure 14: Q-PCR analysis of relative expression of *Runx 2* mRNA. Expression in the OVX-NT group was significantly decreased, compared with that in the Sham group, but increased markedly after 12 weeks of treatment in the BBC-H group (6.39 g/kg·d). Cross-current in the expression of *PPAR γ* , *Notch1* and *Notch2* mRNA. $\Delta p < 0.05$ vs Sham group, $*p < 0.05$ vs OVX-NT group.

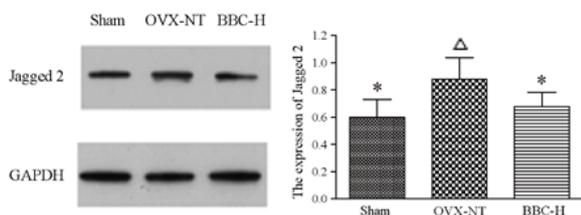


Figure 15: *Jagged2* protein expression of Sham, OVX-NT, and BBC-H groups. GAPDH was measured as a loading control. $\Delta p < 0.05$ vs Sham group. Compared with the OVX-NT group, expression of *Jagged2* was decreased in the BBC-H group. $\Delta p < 0.05$ vs Sham group $*p < 0.05$ vs OVX-NT group.

BBC regulates transcript and protein levels of key layers the Wnt/ β -catenin signaling pathway

BBC treatment enhances Wnt3a and Wnt10b protein expression: Immunohistochemical analyses showed that compared with the Sham group. Expression of these proteins increased in the BBC-H treatment group, compared with the OVX-NT group. The average density was determined using Image-Pro Plus 6.0 Image analysis software, and results are presented in (Figure16).

BBC enhances β -catenin mRNA and protein expression

Total RNA integrity test results revealed successful total RNA extraction (Figure 17). Amplification and melting curves of Q-PCR confirmed the success of PCR. Q-PCR data showed that compared with the Sham group, expression of *GSK-3 β* mRNA was significantly increased ($p < 0.05$) and β -Catenin mRNA was decreased in the OVX-NT group ($p < 0.05$). Conversely, relative to the OVX-NT group, expression of *GSK-3 β* mRNA was significantly decreased ($p < 0.05$) and that of β -Catenin mRNA was significantly increased in the BBC-H group ($p < 0.05$). Consistently, western blot data showed that compared with the Sham group, β -catenin protein expression was significantly lower ($p < 0.05$) in the OVX-NT group, and increased in the BBC-H group ($p < 0.05$).

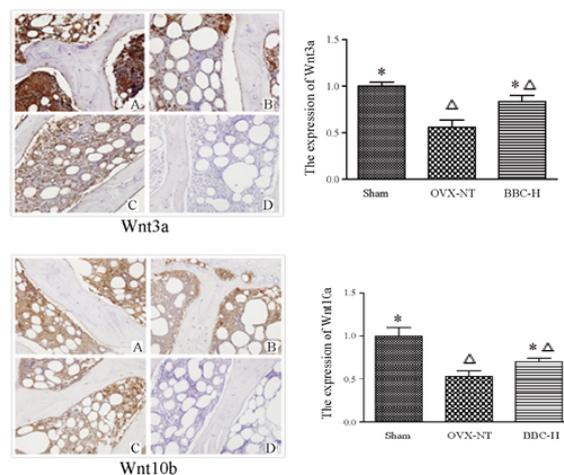


Figure 16: A: Sham group, B: VOX-NT group, C: BBC-H group, D: FOS group. Analysis with Image-Pro Plus 6.0 showed decreased expression of *Wnt3a* and *Wnt10b* proteins in the OVX-NT group, compared with the Sham group. Compared with the OVX-NT group, *Wnt3a* and *Wnt10b* protein expression were increased in the BBC-H group. $\Delta p < 0.05$ vs Sham group, $*p < 0.05$ vs OVX-NT group.

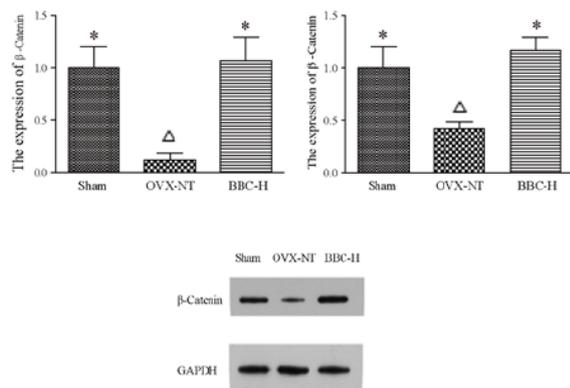


Figure 17: Quantitative assessment of protein expression of β -catenin, relative to the GAPDH group via western blot. Statistical analysis of significant differences in expression between Model and BBC-H groups. $\Delta p < 0.05$ vs Sham group * $p < 0.05$ vs OVX-NT group.

Discussion

Osteoporosis is a significant global health problem, and the development of safe and effective anti-osteoporotic drugs remains an urgent unmet medical need. While Traditional Chinese Medicine (TCM) has been proven by empirical science to be safe and effective in the prevention and cure of osteoporosis, reliable evidence to support their efficacy and safety of TCM formulations is lacking in experimental science. TCM is an exceptional source of potentially useful medications and drug targets. However, the exact mechanisms underlying the pharmacological effects of components of TCM components remain to be established.

In this study, we examined the effects of holistic treatment in ovariectomized rats. Fosamax was selected as the positive control, owing to its documented therapeutic effect on OP. Our results showed that BBC effectively reduces bone mass loss in OVX-NT rats, enhances bone trabecular number and thickness, reduces the degree of separation of trabecular bone, and improves its morphological structure. BBC-H (6.39 g/kg·d) exerted the most pronounced effect on these indices, indicative of the most significant therapeutic effect on osteoporosis and suggesting a dose-dependent curve. Serum aspartate aminotransferase (AST) of rats was not significantly different among BBC, FOS, and Sham groups, signifying that BBC and FOS exert no toxic side effects on liver during the treatment period. ELISA results disclosed that BBC promotes expression of the bone formation related factors estrogen (E2), Bone Gla Protein (BGP) and osteoprotegerin (OPG). Previous clinical studies have reported that BBC improves the symptoms of OP, with no obvious toxicity or adverse reactions over six-month experimental period [21]. The collective findings clearly indicate that BBC effectively improves OP, supporting its potential utility as a treatment/ prevention agent.

Importantly, we observed effects of BBC on several targets of the Notch signaling pathway. After treatment with BBC,

expression of Notch1 and Notch2 mRNA decreased significantly in bone tissue of OP rats. Western blot and immunohistochemical analyses showed that compared with the OVX-NT group, Jagged1 and 2 protein levels were significantly decreased upon BBC treatment, leading to the proposal that BBC may function in blocking the Notch signaling pathway. The biological processes of OP are mainly linked by MSCs, OB, and OC. MSCs are multipotent stem cells that can differentiate into OB, chondroblasts, and lipoblasts, among other cell types. OBs generate bone matrix and fiber, and subsequently, calcified bone matrix, resulting in the formation of bone tissue [22]. Campa et al. [23] demonstrated that the Notch1-Jagged 1, 2 pathways plays a role in MSC osteogenic differentiation. In human MSCs, over expression of Jagged1 and Delta1 through transfection also promotes mineralization and enhances the alkaline phosphatase (ALP) level [24,25]. Another study showed that silk proteins stimulate osteoblast differentiation by suppressing the Notch signaling pathway in MSCs [26,27]. In a hypoxia tolerance test, Hilton and co-workers demonstrated that Notch 1 inhibits osteogenic differentiation of MSCs. The activity of the Notch signaling pathway and the expression of Notch 1 were increased in hypoxia, leading to decreased osteogenic differentiation ability of MSCs, while even under hypoxic conditions, inhibition of Notch 1 promoted MSC differentiation into OB [28]. In addition, knockout of Notch1-3 is reported to stimulate differentiation to OC in bone source macrophages, while the lack of Notch1 inhibits secretion of OPG in OC [29]. Clinically, compared to healthy women, Jagged1 and Notch 1 levels are significantly increased in MSCs of patients with postmenopausal OP [30]. In osteoclast precursors, suppression of the Notch 1 signal promotes differentiation into OC, implying that BBC can suppress bone metabolism through regulation of ALP and OPG (Figure 18).

On the other hand, after treatment with BBC, expression of PPAR γ mRNA decreased and Runx 2 mRNA increased in bone tissue of OP rats. HES and HEY, two of the downstream target genes of Notch signaling, have been shown to reduce the transcriptional activity of Runx2, leading to inhibition of osteogenic differentiation of MSCs [31]. CBF1, the positive adjustment factor of osteogenic differentiation, down regulates the Notch-Hes1/Hey1 signal, resulting in increased expression of Runx2. [32]. Activation of the Notch signal can directly promote adipogenic differentiation and indirectly suppress osteogenic differentiation of MSCs. This may be related to peroxisome proliferator activated receptor γ (PPAR γ), which initiates the whole process of adipogenic differentiation when activated [33]. PPAR γ , a downstream target of Notch signaling pathway, directly reflects the adipocyte differentiation status of MSCs. In rat MSCs with knock out of the PPAR γ gene, adipogenic differentiation is entirely replaced by non-induced osteogenic differentiation [34]. The data indicate that PPAR γ and Notch 1 inhibit osteogenic differentiation of MSCs. Accordingly, we suggest that BBC promotes exerts a therapeutic effect on OP via regulation of PPAR γ and Runx2.

A Water Extract of Benefiting-Bone Capsule Improves Osteoporosis via Transcriptional and Translational Regulation of Key Factors in Notch and Wnt/ β -catenin Signaling Pathways in Ovariectomized Rats

We additionally examined the effects of BBC on the Wnt/ β -catenin signaling pathway. BBC treatment enhanced protein expression of Wnt3a, Wnt10b and β -catenin, indicating activation of Wnt/ β -catenin signaling. *In vitro* experiments have shown that Wnt3a directly induces differentiation of OB and inhibits differentiation of OC [35], while *in vivo*, lack of Wnt3a potentially leads to OP [36]. In mice, defects in the Wnt10 gene resulted in the bone loss and reduction of trabecular bone, while in Wnt10 overexpressing transgenic mice, bone mineral density was significantly increased [37]. Wnt10b protein promotes osteogenesis by increasing Runx2 and inhibiting adipogenesis through reduction of C/EBP α and PPAR γ [38]. β -Catenin is the key protein in the Wnt/ β -catenin signaling pathway. During mouse embryo development, lack of β -catenin completely inhibits osteogenic differentiation [39].

Moreover, BBC induced a significant decrease in GSK-3 β mRNA, a key enzyme of β -catenin phosphorylation, and degradation by the Axin/APC/GSK 3 β complex in the cytoplasm. GSK-3 β enhances expression of the Hes-1 precursor. Cross-talk between Notch and Wnt signaling pathways is partly mediated by phosphorylation of GSK-3 β [40]. During this process, over-expression of the Notch signal promotes adipogenesis and inhibits osteogenesis [41], and additionally downregulates Wnt receptors and downstream targets of Wnt/ β -catenin, consequently inhibiting OB [42].

catenin in the cytoplasm will combine with the APC/Axin/GSK-3 β complex, which, in turn, phosphorylates β -catenin leading to enzymatic degradation. In cases where the Wnt signaling pathways is activated, Wnt proteins combine with surface membrane Frizzled receptor and its co-receptor, LRP5/6, leading to activation of Dsh proteins in the cytoplasm, which inhibits degradation of β -catenin by suppressing phosphorylation activity. Consequently, β -catenin accumulates in the cytoplasm and translocates to the nucleus, where it combines with TCF-4/LEF, promotes the expression of downstream target proteins, Runx2 and Osterix, and upregulates osteogenic differentiation of MSCs [45].

BBC activates the Wnt/ β -catenin pathway through up regulation of Wnt3a and Wnt10b proteins and glycogen synthesis kinase 3 β (GSK-3 β) mRNA and inhibition of Jagged1, 2 protein and Notch 1, 2 mRNA. Moreover, BBC exerts its effects through elevating runt-related transcription factor 2 (Runx 2) and suppressing peroxisome proliferator-activated receptor γ (PPAR γ) mRNA expression.

Conclusion

BBC potentially improves OP and regulate correlation factor through modulation of protein and mRNA expression of key factors in Notch and Wnt/ β -catenin pathways. Further preclinical investigations are required to identify the pharmacological targets of BBC and clarify the relationships between the different signaling pathways involved to optimize its application as an effective treatment option for OP.

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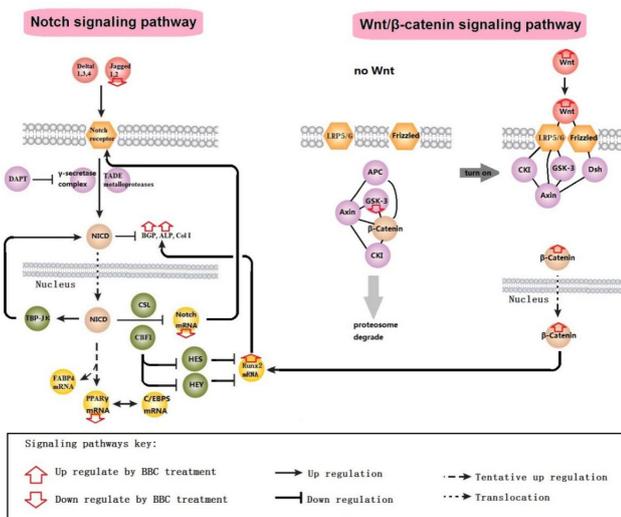


Figure 18: Notch 1, 2, 3, and 4 are four receptors of the Notch family. Notch ligands are transmembrane proteins with conserved structures. Five known Notch ligands exist in mammals: Delta1, 3, and 4 and Jagged1 and 2. Ligand binding to receptors results in successive proteolytic cleavage mediated by TADE metalloproteases and a γ -secretase complex. Cleavage of the Notch receptor results in the release of a constitutively active Notch intra-cellular domain (NICD) that translocates to the nucleus where it binds various transcription complexes and affects the expression of downstream genes [43,44].

The Wnt family is composed of 19 lipidated and glycosylated proteins that play essential roles in diverse processes. Among these, Wnt3a and Wnt10b are closely related to bone metabolism. If Wnt signaling pathways are not activated, β -

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