·药物研究·

Effects of donkey-hide glue reinforcing bone oral solution on expression of type Ⅰ collagen gene and protein expression in ovariectomized rats

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Abstract：**Objective** To investigate the effects of donkey-hide glue reinforcing bone oral solution (DGRBOS) on expression of type Ⅰ collagen gene and protein expression in rats, and explore the mechanism of treating osteoporosis with DGRBOS. **Methods** 36 female SD rats aged 6 months were randomly divided into 3 groups after ovariectomy: A(sham group), B(group of osteoporotic animal model with normal physiological saline), C(treated group of osteoporotic animal model with DGRBOS). Each group included 12 rats. The expression of type Ⅰ collagen gene in bone tissue was detected by real time fluorescence quantitative PCR(FQ-PCR) method and type Ⅰ collagen protein analysis by Western blotting. **Results** Type Ⅰ collagen protein of Group C (OVX + DGRBOS) was significantly higher than B group (ovariectomized + saline group), $P = 0.002$ ($P < 0.01$), there are obvious differences in statistical significance. Type Ⅰ collagen gene fluorescence quantitative PCR (FQ-PCR) results of the B group and C group, $P = 0.004$ ($P < 0.01$), that amplified the differences in the efficiency of a clear statistical significance. **Conclusion** DGRBOS can increase type Ⅰ collagen mRNA expression level and at the same time significantly increase the type Ⅰ collagen content of expression, which is one of the mechanisms that DGRBOS can be treated for Osteoporosis.

Key words: DGRBOS; Collagen Ⅰ; Osteoporosis

阿胶强骨口服液对去卵巢大鼠骨质疏松模型Ⅰ型胶原基因和蛋白表达的影响 郭向飞 倪家骧 沈霖等. 华中科技大学同济医学院附属协和医院,湖北 武汉 430022

摘要：目的 研究阿胶强骨口服液(donkey-hide glue reinforcing bone oral solution, DGRBOS)对去卵巢骨质疏松模型SD大鼠Ⅰ型胶原基因和蛋白表达的影响,探讨DGRBOS治疗骨质疏松的疗效机制。**方法** 6月龄SD大鼠36只,随机分为A组(假手术组),B组(卵巢切除+生理盐水组),C组(卵巢切除+阿胶强骨口服液组),每组12只。6个月后取材检测。采用荧光定量PCR对Ⅰ型胶原基因进行定量分析,采用免疫印迹法对Ⅰ型胶原蛋白进行分析。**结果** C组Ⅰ型胶原蛋白与B组比较明显增高,已接近A组($P < 0.01$),差异有明显的统计学意义。Ⅰ型胶原基因荧光定量PCR(FQ-PCR)结果B组与C组相比,($P < 0.01$),说明扩增效率的差异有明显的统计学意义。**结论** 阿胶强骨口服液可以上调骨组织Ⅰ型胶原mRNA的表达水平,同时显著提高Ⅰ型胶原蛋白表达含量,这是阿胶强骨口服液治疗骨质疏松的机制之一。

关键词：阿胶强骨口服液；Ⅰ型胶原；骨质疏松

Osteoporosis is a disease of the skeletal system. The bones lose density, become brittle and prone to fracture. It is the major cause of bone fractures in older people, especially postmenopausal women. Bone is a complex tissue of which the principal function is to resist mechanical forces and fractures. Bone strength depends not only on the quantity of bone tissue but also on the quality, which is characterized by the geometry and the shape of bones, the microarchitecture of the trabecular

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bones, the turnover, the mineral, and the collagen. Different determinants of bone quality are interrelated, especially the mineral and collagen, Two type of nonenzymatic process are described in type Ⅰ collagen: the formation of advanced glycation end products due to the accumulation of reducible sugars in bone tissue, and the process of racemization and isomerization in the telopeptide of the collagen. This study evaluated the effects of DGRBOS expression of type Ⅰ collagen gene and protein expression in rats by FQ-PCR and Western blotting, in order to elucidate the possible mechanism of DGRBOS involved in osteoporosis treatment.

1 Materials and methods

1.1 Materials

1.1.1 Experimental animals

Thirty six 6-month-old female Sprague Dawley rats (Ⅱ grade, animal qualified No.: medical dynamic characters 20-137) and weighing 320 ± 20 g. DGRBOS (National Medicine Approval No.: Z20000039, Huashi Dan Pharmaceutical Co., Ltd., Xinjiang production and batch number: 2005042107), Trizol solution (purchased from the United States GIBCO company); SYBR Green Ⅰ fluorescent dye (purchased from the United States Biotium company); PROMEGA (purchased from Dafeng Biological Technology Co., Ltd., Wuhan); FTC-2000 (purchased from Shanghai Fengling Biological Technology Co., Ltd.); HERAERUS centrifuge (purchased from Germany).

1.2 Experimental methods

1.2.1 Animal groups and model

Thirty six Sprague Dawley female rats, aged 6 months, were adapted to room and cage environments for at least 7 days before the start of experiments. They were caged singly in a temperature-controlled room (22 ± 1℃) with a 12-h light/dark cycle, maintained on a commercial pelleted chow diet (purchased from the Tongji Medical College animals Experimental Center) with free access to water. A week after the acclimatization were divided into three groups: Group A were sham operated and given intragastric administration of normal sodium; group B were oophorectomized and given intragastric administration of normal sodium; group C were oophorectomized and given intragastric administration of DGRBOS. Group B and C were given two percent Pentobarbital sodium 40 mg/kg to intra-abdominal anesthesia, and then were given blunt abdominal muscle separation, peritoneal incision into the abdominal cavity, ligation and complete removal of bilateral ovarian and carefully stop bleeding, layer by layer suture. They were given an intramuscular injection of penicillin (80000 units/only), to prevent infection. Group A were given the same disposal except ovarian hypodesis and exclusion. Animals were killed after 6 months.

1.2.2 Drug intervention

Each animal in Group C was given by intragastric administration, in accordance with human and animal drug dose conversion method[1], 2 ml DGRBOS each time, twice a day. Accordingly each animal in Group A and B was given 2 ml normal saline each time by intragastric administration, twice a day.

1.2.3 Specimen processing

The rats were killed at ice, quickly removed the femoral heads and then quick triturate into powder, shift in homogenizer and add TRIzol (2 ml TRIzol per 100 mg femoral head). Freezing homogenate, and then take homogenate into EP tubes, incubate in 15-30 ℃ for 5 min, add chloroform ((0.2 ml isopropanol/1 ml TRIzol); tight the cover and shake with force for 15 seconds and incubate them in 15-30 ℃ for 2 ~ 3 min, centrifugate 12000 r/min in 4 ℃ for 15 min, take supernatant to new EP tubes, add DEPC water to dissolve RNA, preserve them in −80 ℃ for employ.

1.2.4 Primer design

Primer sequences were from Gene bank database, in accordance with the relevant literature, by the Sanbaisheng Company in Shanghai.

1.2.5 Reverse transcription reaction

With the help of reverse transcriptase M-MLV, mRNA was reversed and transcripted into cDNA molecules. RT reaction system is as follows: H_2O 5.5 μl、Oligo(dT_{18}) 1.0 μl、TRNA 6.0 μl、RNasin 0.5 μl、5 × buffer 4.0 μl、10 mmol/L dNTP 2.0 μl、RTase 1.0 μl. Conditions: 42℃ for 60 min, 95℃ for 5 min.

Table 1 COL I gene primer sequence

Number	Name	Sequence(5′ > 3′)	Length	(GC%)	Tm(℃)
0711-108	COLI FW	GGCTTCTTCAAACCACTGCTTT	22	50.0%	56.7
0711-109	COLI RV	AAAGTCATAGCCACCTCCGCTG	22	45.8%	56.7
0711-110	β-ACTIN FW	TCCTAGCACCAT GAA GATC	19	48.0%	57.7
0711-111	β-ACTIN RV	AAACGCAGCTCAGTAACAG	19	50.0%	54.4

1.2.6 Fluorescence quantitative PCR(FQ-PCR)

FQ-PCR reaction system is as follows: cDNA 1 μl、Buffer 10 × 5 μl、$MgCl_2$ (25 mmol/L) 7 μl、dNTP 10 mmol/L 1 μl、F (20 pmol/μl) 0.8 μl、R (20 pmol/μl) 0.8 μl、SYBR Green Ⅰ 1 μl、Taq enzyme (5 U/μl) 0.5 μl. Conditions: 94℃ for 3 min, (94℃ for 30 sec, 53℃ for 30 sec, 72℃ for 30 sec) 50 cycles.

1.2.7 Detection of COL I protein by Western blotting

Protein samples were separated by SDS-PAGE and transferred to ECL nylon membrane, COL I antibodies were incubated with 100 mg of oocyte extract in 5 mmol/L KCl or 50 mmol/L NaCl, or 100 mg of the BioRex 0.5 mol/L fraction in 100 mmol/L NaCl, prepared as above, for 2 h at 4 ℃ with rotation. The immuno precipitations were washed three times with Buffer A (0.1 mol/L) and assayed by western-blot analysis.

1.2.8 Application of SYBR Green Ⅰ fluorescent dye technology to real-time PCR reaction to access to the standard curve each group, computer analysis Ct value. The films were developed, and the stained bands of the colloxylin membrane were scanned and analyzed using the gel electrophoresis imaging analytical system, the gray value of the band (area × average gray value) represented the amount of protein expression. Statistical analysis: The data were statistically analyzed by the second author using SPSS 12.0 software. The measurement data were expressed as Mean ± SD, and the differences were compared using Chi-square test.

2 Results

2.1 COL I mRNA expression

Table 2 lists every Ct value which was calculated by computer. Calculation method is as follows: Under test sample relative value = 2 − (△Ct β-actin − △Ct under test samples), △Ct = Ct negative control − Ct under test samples, △△Ct = △Ct β-actin − △Ct to be under test samples = Ct Test samples − Ct β-actin. collagen Ⅰ gene expression has a statistically significance between Group B and Group C ($P = 0.004$).

Table 2 Collagen Ⅰ and β-actin FQ-PCR Ct value($\bar{x} \pm s$)

组别	collagen Ⅰ Ct value	β-actin Ct value	−△△Ct	$2^{-\triangle\triangle Ct}$
A	27.4523 ± 1.4657	26.5024 ± 1.6259	0.9301 ± 0.0328	1.8325 ± 0.1942
B	25.5713 ± 2.0038	25.1147 ± 2.1517	0.5007 ± 0.1123	1.3019 ± 0.1027
C	27.1245 ± 1.2672	26.5864 ± 1.1563	0.6784 ± 0.2964	1.5334 ± 0.1210*

Compared with Group B, * $P < 0.01$

Note: The Ct(cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold, i.e. exceeds background level. Ct levels are inversely proportional to the amount of target nucleic acid in the sample, i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample.

2.2 COL I protein by Western blotting

COL I protein showed a statistically significance between Group B and Group C ($P < 0.01$).

3 Discussion

The typical characteristic of Osteoporosis (OP) is reduced bone mass, degradation of bone microstructure and bone fracture easily. It is commonly present in postmenopausal women, the elderly and patients with multiple chronic diseases. Modern medicine has in-depth study on the OP to the molecular level, and considers that the basic pathological mechanisms of OP is a coupling

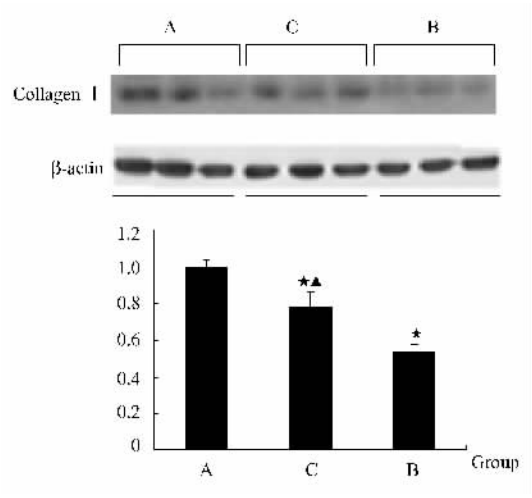

Fig.1 Collagen I protein western blot result
vs A group ★ $P < 0.01$;vs B group ▲ $P < 0.01$

imbalance in the process of resorption of bone metabolism and bone formation, leading to the body's metabolic imbalance of calcium and phosphorus, bone density gradually reduced, and triggers the clinical symptoms. Rats used for the present study is the most commonly used animal OP model. In ovariectomized rats, bone turnover of the spongy bone accelerated, bone mass reduced, bone strength decreased, whose features is similar to the normal bone loss in postmenopausal status ; Although the female rats with Ovarian removal were given a suitable alternative to estrogen, it does not increase the trial of bone turnover and loss, this is in line with the response of postmenopausal women with estrogen replacement[2] . It is generally believed that the copy of osteoporosis model in postmenopausal women should choose the 6-9 month-old female rats, three months after dual-line ovariectomized, Osteoporosis can be established[3] .

Type Ⅰ collagen metabolism may be a good indicator to reflect the level of bone metabolism, bone mineral density also changes, and Type Ⅰ collagen is in good correlation with vertebral and hip fracture rate, its sensitivity, specificity and stability of the bone were higher than previous biochemical indicators, it is a great value to determine drug efficacy and mechanism of fracture risk prediction in early diagnosis and treatment of osteoporosis[4] .

Chinese medicine practitioners believe that osteoporosis belongs to " bone paralysis " areas. " Kidney being the congenital base of life, kidney storing essence, reinforcing kidney to replenish marrow. " Bone growth, development, strong or weak, is related to the Rise and Fall of kidney essence. Spleen is weakness, loss the function of gasification, cardinalate stagnation, leading to bone paralysis. In addition, the spleen and kidney are virtual, reconfront Waixie invasion, and produce cold hygrosis, Qizhixueyu and so on, which is the pathogenesis of this disease. Therefore, the disease lesions in the bones mainly involving spleen and kidney, is the syndrome of benxubiaoshi. Chinese medicine has achieved certain progress in the prevention and treatment of osteoporosis research. DGRBOS is composed with gelatina nigra, concha ostreae, medlar, astragalus mongholicus and codonopsis pilosula, which plays the role of replenishing liver and kidney and which take a part in essence replenishment and strengthen bone. DGRBOS promotes osteoblast cells and cartilage cells into mitosis, blood vessel endogeny and bone constructive metabolism through the bone-VEGF and FGF-2[5] .

The results showed that DGRBOS can increase type Ⅰ collagen mRNA expression level and at the same time significantly increase the type Ⅰ collagen content of expression, which is one of the mechanisms in which DGRBOS has therapeutic action in osteoporosis.

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