Effects of *Drynariae Rhizoma* Total Flavonoids on *Smad1* and *Smad5* mRNA Expression in Osteoporotic Rats

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Abstract: This study aimed to investigate effects of the total flavonoids in *Drynariae Rhizoma* on the *Smad1* and *Smad5* mRNA expression in ovariectomized rats. A total of 60 SD rats were divided into the normal, blank control, and premarin-treated groups as well as three *Drynariae Rhizoma* total flavonoid-treated groups (with $n = 10$ per group). The flavonoid-treated groups received high, moderate, and low doses of *Drynariae Rhizoma*. All rats were ovariectomized, except for those in the normal group. The normal and blank control groups were fed with standard feed for 24 weeks. The flavonoid-treated groups were ovariectomized at 12 weeks and fed with *Drynariae Rhizoma* in three different concentrations for the remaining 12 weeks. The premarin-treated group was similarly ovariectomized at 12 weeks but fed with premarin for the remaining 12 weeks. All rats were sacrificed, and their right femur bones were collected for detecting *Smad1* and *Smad5*. The *Smad1* and *Smad5* expression of the blank control group was 40% and 59.5%, respectively, of the normal levels ($P < 0.05$ for both). By contrast, all *Smad1* and *Smad5* expression was significantly increased by *Drynariae Rhizoma* treatment, regardless of the dose, as compared with the blank control group ($P < 0.05$). *Smad5* gene expression was significantly increased by the moderate dose of *Drynariae Rhizoma* ($P < 0.01$). The total flavonoids in *Drynariae Rhizoma* promoted *Smad1* and *Smad5* gene expression in the bone marrow of ovariectomized rats, particularly the moderate dose of *Drynariae Rhizoma* total flavonoids.

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1. Introduction

Smad is a transcription factor of the transforming growth factor (TGF)-β family; this protein is important for intracellular signal transduction (Cai and Deng, 2007). Silk threonine kinase receptors types I and II may be transmitted as downstream signals of Smad1 and Smad5, which are closely associated with bone generation.

The effects of *Drynariae Rhizoma* on Smad4 expression have been reported in ovariectomized rats (Huang et al., 2006). However, the effects of *Drynariae Rhizoma* total flavonoids on *Smad1* and *Smad5* expression have not been investigated to date, particularly not in osteoporotic rats. Pure *Drynariae Rhizoma*, its total flavonoids, or its preparation as traditional Chinese medicine compound have been reported to affect bone tissue gene expression, but not *Smad* mRNA expression (Han et al., 2006).

This study investigated the mRNA expression of the Smad genes *Smad1* and *Smad5* after feeding ovariectomized rats with the total flavonoids from *Drynariae Rhizoma*. This study attempted to determine the molecular mechanism of *Drynariae Rhizoma* total flavonoids for rat models of osteoporosis (OP).

2. Materials and methods

2.1 Animals and grouping

A total of 60 female SD rats were bought from the Animal Experiment Research Center of Zhejiang Chinese Medical University. The rats were approximately aged 12 weeks old and weighed (240 ± 20) g. The rats were divided into six groups ($n = 10$ per group): the normal group (not ovariectomized), the blank control group (ovariectomized but without treatment), the premarin-treated group, and three *Drynariae Rhizoma* total flavonoid-treated groups that received high, moderate, and low doses.

Each rat was anesthetized by an intraperitoneal injection of 2% pentobarbital (dosage, 35 mg/kg), and a 2 cm-long incision was performed along the middle abdominal line. Only a small piece of adipose tissue was cut with a weight nearly equal to the ovary, except for the sham-operated group (normal group). The other groups were ovariectomized, and their abdominal walls were treated with two-level sutures after an intraperitoneal injection of 200 000 units of penicillin. Intraperitoneal injection of 200 000 units of penicillin was carried out 3 days after operation. Normal feeding was started after 12 weeks; *Drynariae Rhizoma* was introduced during the 13th

week. Each group received different treatments. The normal and blank control groups continued to be fed with standard feed for the remaining 12 weeks of the experiment. The flavonoid-treated groups were fed with standard feed combined with different concentrations of *Drynariae Rhizoma* total flavonoids (high, moderate, and low doses) for the remaining 12 weeks. The premarin-treated group was fed with standard feed and administered with the premarin suspension for the remaining 12 weeks (dosage, 10 ml/kg). The different concentrations of each treatment are listed in Table 1.

Table 1 The concentrations of drug executives on each group

Drugs	Concentrations (ml-1)
Drynariae Rhizome Total Flavones	
Low dose group	0.054g
Middle dose group	0.108g
High dose group	0.216g
Premarin see conjugated estrogen	9.375ug

The rats were weighed every four weeks, and the treatment dosage was adjusted according to their weights. After 24 weeks, all animals were anesthetized with 2% pentobarbital and sacrificed; their right femurs were collected for further analysis. The soft tissue was removed and stored at -70 ℃.

2.2 Real-time (RT)-PCR

The left femoral bone was removed using gouge forceps after thawing each sample. The bone marrow was carefully collected from each sample and transferred into 1.5 ml centrifuge tubes. Each bone marrow sample was flushed with 1 ml saline and centrifuged at 12 000 rpm for 10 min. The total RNA was extracted using Trizol according to previous methods (Pang et al., 2004). Reverse transcription in cDNA was performed using the extracted RNA as template according to the literature (Xu et al., 2009). The RT-PCR reaction system contained: dNTPs, 1 μl; 10× buffer, 5 μl; *Taq* DNA polymerase, 1 μl; forward primer, 1 μl; reverse primer, 1 μl; cDNA template, 2 μl; and H_2O, 39 μl. (The primer sequences are presented in Table 2.) The following PCR profile was used: 94 ℃ for 3 min; 30 cycles of 94 ℃ for 40 s, 55 ℃ for 30 s, and 72 ℃ for 30 s; 70 ℃ for 5 min. The PCR products were detected by 1.5% agarose gel electrophoresis.

Table 2 Primers used in this study

Names	sequence	Product length (bp)
Smad1	L:5'-acgag gaacc aaaac actgg-3	165
	R-tccgg ttaac attgg agagc	
Smad5	L-acgag gaacc aaaac actgg	166
	R-tccgg ttaac attgg agagc	
β-actin	L-ttgat gtcac gcacg atttc	213
	R-tgtcc ctgca tgcct ctggt	

2.3 Fluorescent quantification using RT-PCR (Qiu, 2000)

The PCR system for quantification contained: SYBR Green fluorescent dye, 25 μl; dNTPs, 0.5 μl; 10× buffer, 2.5 μl; *Taq* DNA polymerase, 0.5 μl; forward primer, 0.5 μl; reverse primer, 0.5 μl; reverse transcription production, 2 μl; H_2O, 18.5 μl. (The primer sequences are presented in Table 2.)

The following quantitative PCR profile was used: 94 ℃ for 3 min; 45 cycles of 94 ℃ for 30 s, 55 ℃ to 95 ℃ for 30 s, and 72 ℃ for 30 s. The fluorescent signals were measured at 72 ℃ (during the extension step of each cycle). The melting curve was measured from 55 ℃ to 95 ℃ with a ramp increase of 0.5 ℃/s for each cycle.

2.4 Statistical analysis

The results for the relative quantification were analyzed by $2^{-\Delta\Delta C(t)}$. Data were expressed as the mean ± standard error. The SPSS (version 13.0) statistical package was used for the single-factor ANOVA and data processing. $P < 0.05$ was considered statistically different, whereas $P < 0.01$ was considered significantly statistically different.

3. Results

During the experiment process, one rat died in the premarin-treated group, whereas the rats in the blank control group and the high-dose *Drynariae Rhizoma* total flavonoid-treated group were all alive. The results of non-fluorescent PCR and electrophoresis after the processing by the Tanon GIS-2009 gel imaging system are shown in Figs. 1 and 2.

Figure1. The electrophoresis result of Smad1. 1,2 normal group; 3,4 blank control group; 5,6 premarin see conjugated estrogen group; 7,8 high dose group; 9,10 middle dose group; l1,12 low dose group.

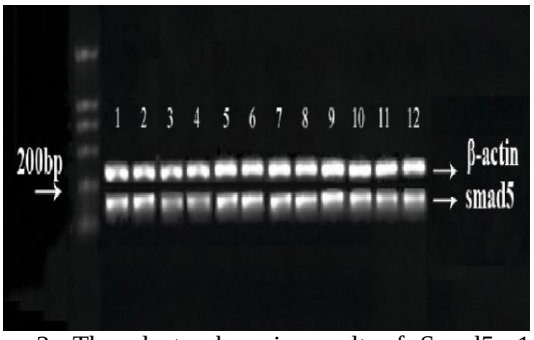

Figure2. The electrophoresis result of Smad5. 1, 2 normal group; 3,4 blank control group; 5,6 premarin see conjugated estrogen group; 7,8 high dose group; 9,10 middle dose group; l1,12 low dose group.

The mRNA expression of *Smad1* in the blank control group was 40% of that of the normal group, thereby indicating a statistical difference ($P < 0.05$). Premarin treatment restored normal *Smad1* mRNA expression, which was significantly different from that of the blank controls ($P < 0.05$). Compared with the blank control group, the increased mRNA expression of *Smad1* was statistically different for each dose of the *Drynariae Rhizoma* total flavonoids ($P < 0.05$). The *Smad1* mRNA expression of the groups with high and moderate doses of *Drynariae Rhizoma* total flavonoids were not significantly different from that of the premarin-treated group ($P > 0.05$). However, the mRNA expression of *Smad1* was significantly different between the low-dose flavonoid-treated group and the premarin-treated group ($P < 0.05$).

The mRNA expression of *Smad5* in the blank control group was 59.5% of that of the normal group, thereby indicating their statistical difference ($P < 0.05$). Premarin treatment restored normal *Smad5* mRNA expression, which was significantly different from that of the blank controls ($P < 0.05$). Compared with that of the blank control group, the mRNA expression of *Smad5* on each dose of *Drynariae Rhizoma* plus total flavone group increased and a statistical difference was observed ($P < 0.05$). The mRNA expression of *Smad1* was significantly statistically increased after treatment with high and moderate doses of *Drynariae Rhizoma* total flavonoids ($P < 0.01$), whereas that of *Smad5* on the low dose group statistically increased ($P < 0.05$). Compared with that of the premarin group, no significant difference on the mRNA expression of *Smad5* on the high and low dose groups was observed ($P > 0.05$). However, a significant difference occurred on the mRNA expression of *Smad5* on the moderate dose group ($P < 0.05$).

4. Discussion

The possible harm caused by OP becomes a serious concern as a population ages because OP may induce fractures (Li et al., 2008). The occurrence of fractures (particularly hip fractures) in the elderly may seriously affect their quality of life or even lead to death. Approximately 84 million people in China are affected by OP (including bone loss) to date, based on a large number of epidemiological studies (Cai and Deng, 2007). This figure accounts for 6.0% of the total population, with the highest morbidity in postmenopausal women. Therefore, the prevention and treatment of OP is an urgent and difficult clinical objective (Bornstein et al., 2007; Li and Zhang. 2004).

Drynariae Rhizoma is commonly used to treat bone damage; this form of traditional medicine has begun to receive considerable attention (Wang et al., 2008). The effects of *Drynariae Rhizoma* on expression in ovariectomized rats have been analyzed, but previous studies did not consider the total flavonoids in *Drynariae Rhizoma*. In addition, previous studies only focused on *Smad4* mRNA. To the best of our knowledge, the effects on *Smad1* and *Smad5* mRNA have not been previously reported. The effect of pure *Drynariae Rhizoma*, its total flavonoids, and its derivative Chinese medical compounds on gene expression in bone tissues have been previously described (Liu et al., 2008; Liu et al., 2008), but these studies did not include the *Smad* mRNA expression.

The TGF family includes TGF-β, activins, inhibin, bone morphogenic proteins, the Mullerian inhibitory substance, growth or differentiation proteins (Massague, 1998), decapentalegic gene products, Vgl, nodal, and dorsalin. These growth factors are signaling molecules related to the regulation and control of cell proliferation, differentiation, and growth. Furthermore, TGFs could stimulate extracellular matrix formation. The Smad family represents a novel and important gene family involved in the TGF-β signal transduction pathway of vertebrates (Derynck and Feng, 1997). This gene family has unique functions in signal transduction (Mao et al., 2004).

The end of the Smad C-terminal functional

domain contains a conserved phosphorylation site S(V/M)S (Flanders et al., 2002), which can recognize type I receptors and initiate phosphorylation. The interaction of specific Smad proteins and their receptors involves the L3 loop structure in their MH2 functional domain (Hata et al., 2002). Given that this interaction is transient, Smad proteins are released by their receptors after phosphorylation. Specific Smad proteins induce mutations in phosphorylation sites, steadily bind to their receptors, and produce a dominant-negative effect. Phosphorylated Smad proteins and certain common Smad proteins may form complexes. These complexes are transferred to the nucleus to regulate the responses of target genes during TGF-β signaling (Tylzanowski et al., 2001).

Smad4 has relatively low isogeny, as compared with the other members of the Smad family (Itoh and ten Dijke, 2007). Its C-terminal function domain does not contain phosphorylation sites. Smad4 cannot interact with TGF-β or BMP type I receptors and cannot be phosphorylated, but it can form a stable heterotrimer with other members of the Smad family (Li and Zhang, 2004). Heterogeneous trimer formation is necessary for TGF-β signal transduction because Smad4-deficient cells cannot respond to TGF-β signaling. Thus, cellular responses to TGF-β may be reconstructed after transfection of the wild-type *Smad4* gene. Similarly, mutations affecting trimer formation may inactivate Smad4. This phenomenon suggests that the interaction between the MH2 functional domain is significant for signal transduction (Xu et al., 2006).

Smad6 and Smad7 have different structures from the other Smad proteins. Their C-terminal functional domains lack phosphorylation sites. Moreover, they do not have conserved MH1 functional domains. Instead, only a MH2 functional domain is present, which has higher homology to other Smad proteins (Tsumaki and Yoshikawa, 2006). The overexpression of Smad6 and Smad7 may prevent signal transduction by members of the TGF-β superfamily; thus, these proteins are known as inhibitory Smads (Shang et al., 2008).

In this study, the expression of *Smad1* and *Smad5* was evidently decreased, as compared with the normal group. The significant expression of *Smad1* mRNA in the blank control group was 40% that of the normal levels ($P < 0.05$). After premarin treatment, the expression of *Smad1* mRNA returned to normal levels; these levels were statistically significant, as compared with the blank controls ($P < 0.05$). Compared with the normal group, the expression of *Smad1* mRNA was increased by treatment with high, moderate, and low doses of *Drynariae Rhizoma* total flavonoids. The blank control group and the flavonoid-treated groups were significantly different ($P < 0.05$). This result implied that all doses of *Drynariae Rhizoma* total flavonoids may have a therapeutic effect on OP.

Compared with the premarin-treated group, the groups treated with high and moderate doses of *Drynariae Rhizoma* total flavonoids were not significantly different ($P > 0.05$). By contrast, the low-dose flavonoid-treated group was significantly different ($P < 0.05$). This finding suggested that the therapeutic effects of high and moderate doses of *Drynariae Rhizoma* total flavonoids were similar to that of premarin, whereas the lower dose was relatively poor.

The *Drynariae Rhizoma* total flavonoids could increase the expression levels of *Smad1* and *Smad5* mRNA in the bone marrow microenvironment. The increased expression may be used as a mechanism to promote bone formation and restoration.

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