Original article

Anti-hypertensive and cardioprotective effects of a novel apitherapy formulation via upregulation of peroxisome proliferator-activated receptor-α and -γ in spontaneous hypertensive rats

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ARTICLE INFO

ABSTRACT

Ventricular remodeling is associated with many heart diseases, and ventricular remodeling induced by hypertension can be fatal independent of hypertension. In this study, we prepared a novel apitherapy formulation, designated Bao-Yuan-Ling (BYL), which contained propolis, royal jelly, and bee venom, to treat spontaneous hypertensive rats (SHRs). We then evaluated the pharmacology of BYL and the potential mechanisms through which BYL affects hypertension and ventricular remodeling. We found that BYL treatment could reduce blood pressure in SHRs. Thereafter, we found that BYL treatment reduced serum levels of angiotensin II, endothelin 1, and transforming growth factor-β and improved the myocardial structure. Moreover, the results of quantitative real-time polymerase chain reaction indicated that BYL treatment could upregulate the mRNA expression of peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ. Thus, we could conclude that BYL had hypotensive and cardioprotective effects in SHRs, potentially through improvement of myocardial energy metabolism.

1. Introduction

Hypertension, which is associated with high mortality worldwide, can cause damage to some target organs, including the brain, heart, and kidney (Mensah et al., 2003). Left ventricular remodeling induced by hypertension is an independent cause of cardiac failure, sudden death, and myocardial infarction (Purushothaman et al., 2011). During the process of ventricular remodeling, myocardial hypertrophy and myocardial fibrosis occur (Ishizu et al., 2014), and energy metabolism, including carbohydrate and lipid metabolism, can be altered. Peroxisome proliferator-activated receptors (PPARs) participate in energy metabolism by regulating the transcription of certain genes (Kota et al., 2005). Although PPARα and

PPARγ have different functions in lipid metabolism, both proteins play important roles in lipid and glucose metabolism in the cardiovascular system (Schiffrin, 2005; Van Bilsen and Van Nieuwenhoven, 2010). In addition, because PPARα and PPARγ regulate the inflammation process, which affects cardiovascular diseases, the roles of PPARα and PPARγ in inflammation regulation in the cardiovascular system have received considerable attention. Importantly, PPARα and PPARγ could be potential therapeutic targets for the treatment of cardiovascular diseases (Van Bilsen and Van Nieuwenhoven, 2010).

Functional foods have been developed to protect against cardiovascular diseases and treat hypertension (Majumder and Wu, 2015). To date, various bee products have been shown to exert cardioprotective effects through different mechanisms. Additionally, propolis, royal jelly, and bee venom are well-known healthcare foods and have been extensively studied in apitherapy. Although the chemical composition of propolis is affected by the origin and plant resources (Mishima et al., 2005), the properties of propolis always include antibacterial, anti-inflammatory, and antioxidative effects (Burdock, 1998). Moreover, propolis has been shown to have hypotensive and cardioprotective effects (Gogebakan et al., 2012; Majiene et al., 2006; Silva et al., 2015). Royal jelly is effective for protecting the reproductive system and

exhibits anti-aging, anti-inflammatory, and antibacterial effects (Ramadan and Al-Ghamdi, 2012). A recent study showed that royal jelly administration has protective effects on paclitaxel-induced cardiotoxicity (Malekinejad et al., 2016). Additionally, gastrointestinal enzyme production by royal jelly shows antihypertensive effects in vitro (Matsui et al., 2002). Bee venom is a natural biological toxin; in addition to its traditional use as an anti-inflammatory, analgesic, immunoregulatory, and anticancer agent (Hwang et al., 2015; Oršolić, 2012), bee venom can also elicit vasodilation effects and promote cardiac inotropy (Hutcheson and Griffith, 2000; Saleh and Saleh, 2011).

When choosing a type of bee product, consumers may select a variety of products as a dietary supplement; however, the optimal combination of components has not yet been determined. Thus, in this study, we aimed to prepare a novel apitherapy formulation, designated Bao-Yuan-Ling (BYL), which contained propolis, royal jelly, and bee venom. We then determined whether this novel formulation could play a role in cardiovascular diseases. Our results provide important insights into the effects of BYL on hypertension and myocardial remodeling in spontaneous hypertensive rats (SHRs).

2. Materials and methods

2.1. Preparation of drugs

The novel apitherapy formulation BYL was prepared from propolis, royal jelly, and bee venom. Crude propolis collected from poplar by worker bees (*Apis mellifera* L.) was kindly supplied by Shenfeng Science and Technology Development Co., Ltd. (Fuzhou, China) and was dissolved in 80% alcohol at 1:4 (g:mL), followed be ultrasonic pretreatment at 40 kHz for 20 min. Thereafter, the propolis was extracted at 60 °C for 5 h and then at room temperature for 2 days. The extract was filtered by vacuum filtration and then concentrated at 60 °C. After removing the wax, the ethanol-extracted propolis was dried under vacuum at 55 °C to a constant weight. The total flavonoid content was 302.16 ± 2.33 mg/g, as measured by the colorimetric method (Woisky and Salatino, 2015). Bee venom from honeybees (*A. mellifera* L.) kept at Chunyuan bee yard (Fuzhou, China) was collected using an electronic bee venom collector (QF-1 type; Qingsong Electronic Appliance Co., Ltd., Fuzhou, China) from May to June. Crude bee venom was dissolved in deionized water, and impurities were removed after centrifugation at $4000g$ at 4 °C for 15 min. Thereafter, the supernatant was filtered with a 0.45-μm membrane and was freeze-dried with a lyophilizer (Four-ring Science Instrument Plant Beijing Co., Ltd, China). Dried bee venom was then stored at −20 °C until use. Royal jelly from worker bees (*A. mellifera* L.) collected from Tongxiang city in March was dissolved in 95% alcohol (1:4, g:mL) for 3 h at 40 °C, and the mixture was then centrifuged at $4000g$ for 30 min. Thereafter, the supernatant was collected, and the deposit was extracted two more times in accordance with the above method. Finally, the above three volumes of filtrate were combined and concentrated with rotary evaporation. In the mixture, the composition ratio was 1000:1000:1 (ethanol-extracted propolis: royal jelly: bee venom, mass ratio). To ensure that the components were completely suspended, we used a sodium carboxymethyl cellulose (CMC) aqueous solution, which is commonly used in the pharmaceutical industry as an excipient, as the vehicle. Additionally, the contents of Valsartan capsules (Beijing Novartis Pharmaceutical Co., Ltd.) were used as a positive control.

2.2. Animals and groups

Fifty male SHRs (weighing 180–220 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Ten male Wistar rats (weighing 180–220 g) were purchased from Shanghai Shanghai Slac Laboratory Animal Co., Ltd. According to the Guidelines of the International Committee on Laboratory Animals, rats were maintained in an environmentally controlled room on a 12-h light/dark cycle with a relatively stable ambient temperature of 23–27 °C and a humidity of 52–58%. All rats had free access to food and water. The rats were acclimated to the feeding schedule for 1 week before the formal trial. Subsequently, rats were divided into six groups, as listed in Table 1.

2.3. Measurement of blood pressure

The tail artery systolic blood pressure (SBP) was measured using an automatic measuring instrument for the tail artery (BP2010-A; Softron Beijing Biotechnology Co., Ltd.) at the beginning of the treatment period and at the end of the first, third, and fifth weeks during the experiment. For the instrument, the operating temperature was set at 37 °C, and the maximum pressure was 220 kPa after collecting records from three parallel tests.

2.4. Preparation of blood and cardiac samples

Rats were sacrificed after 5 weeks of treatment. Blood samples were collected from the aorta abdominals and then stored in centrifuge tubes at room temperature for 2 h. Next, the blood samples were centrifuged at 3500 rpm at 4 °C for 15 min for collection of supernatants. Parameters, including angiotensin II (Ang II), endothelin 1 (ET-1), and transforming growth factor-β (TGF-β), were evaluated by enzyme-linked immunosorbent assays (ELISAs) following the instructions of the ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd.).

After collection of blood samples, whole hearts were removed and placed in saline (cooled to 4 °C) in order to clean the blood. Then, a piece of cardiac tissue (0.1 g) was obtained and stored in liquid nitrogen. The remaining cardiac tissue was fixed in 4% paraformaldehyde solution.

2.5. Histopathology

Paraffin sections were prepared follow standard protocols. Briefly, the fixed tissues were dehydrated through a series of ethanol concentrations (50%, 70%, 80%, 90%, 95%, and 100%) for 1 h. Thereafter, samples were transparentized with dimethylbenzene and embedded in paraffin blocks for subsequent steps. Paraffin-embedded sections were cut to 5 μm thickness and stained with hematoxylin and eosin (HE) and Masson stain.

2.6. Real-time polymerase chain reaction (PCR)

Total RNA from myocardial tissue was extracted with TransZol UP (Beijing TransGen Biotech Biotechnology Co., Ltd.) according to the manufacturer's instructions, and reverse transcription was then performed after RNA quality identification. Real-time PCR was carried out as described for the kit (Takara Biotechnology Co., Ltd.) on a CFX384 Touch Real-Time PCR Detection System

Table 1
Animal groups and treatment.

Group name	Drug	Dose
Control	CMC	1 mL/100 g body weight
Model	CMC	1 mL/100 g body weight
BYL-L	BYL	0.5 g/kg body weight
BYL-M	BYL	1 g/kg body weight
BYL-H	BYL	2 g/kg body weight
Positive	Valsartan	7.2 mg/kg body weight

(Bio-Rad Laboratories, Inc., Hercules, CA, USA). Primers were designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Table 2). The relative value of mRNA expression was measured by the $2^{-\triangle\triangle ct}$ method.

2.7. Statistics

All data are expressed as means ± standard deviations (SDs). Normally distributed data were analyzed using one-way analysis of variance (ANOVA) followed by least significant difference tests. Data that were not of normal distribution were analyzed by one-way ANOVA followed by Dunnetts' T3 test.

3. Results

3.1. Effects of BYL on blood pressure

Fig. 1 shows changes in SBP during the 5-week treatment period in rats. Briefly, there were no significant differences among SHR groups at the beginning of the experiment, indicating that the baseline blood pressure of different SHR groups was the same. In the model group, SBP increased consistently over the 5-week period. This trend was not reversed in any treatment group in our study; however, drug intervention suppressed this increase, and all SHR groups showed significant decreases in SBP compared with that in the model group ($p < .05$). Compared with that in the model group, SBP was reduced by approximately 9.873 ± 1.788 ($p < .05$), 7.437 ± 5.028 ($p > .05$), and 7.540 ± 3.806 ($p < .05$) mmHg, respectively, in the BYL-L group; 16.037 ± 1.429 ($p < .01$), 15.613 ± 2.193 ($p < .01$), and 15.550 ± 1.670 ($p < .01$) mmHg, respectively, in the BYL-M group; and 5.493 ± 0.451 ($p < .01$), 12.082 ± 5.343 ($p < .01$), and 9.342 ± 5.914 ($p < .05$) mmHg, respectively, in the BYL-H group. Interestingly, the BYL-M group exhibited better therapeutic effects in terms of blood pressure reduction than did other BYL treatment groups.

### 3.2. Effects of BYL on blood pressure indexes in serum

All BYL treatment groups showed lowered tested serum indexes, indicating hypertension (Table 3). Moreover, we observed concentration-dependent reductions in all tested indices in the BYL treatment groups. Although the valsartan group (positive control group) showed better effects after Ang II and ET-1 treatment than after BYL treatment, the decrease in TGF-β in the positive control group was lower than those in the BYL-M and BYL-H groups. Moreover, there was no significant difference in the content of TGF-β in the BYL-M and BYL-H groups compared with that in the control group.

3.3. HE is staining

Myocardial fibers in control group were arranged closely and neatly with no abnormalities (Fig. 2A). For model rats, some

Table 2
Primers used in real-time PCR.

Gene name	Accession No.	Primers
PPAR-α	NM_013196	Forward: 5′-GCGTACGACAAGTGTGATCG-3′
		Reverse: 5′-AAACGGATTGCATTGTGTGA-3′
PPAR-γ	NM_001145367	Forward: 5′-TGTGGACCTCTCTGTGATGG-3′
		Reverse: 5′-AGCTCTTGTGAACGGGATGT-3′
β-Actin	NM_031144	Forward: 5′-GGAGATTACTGCCCTGGCTCCTA-3′
		Reverse: 5′-GACTCATCGTACTCCTGCTTGCTG-3′
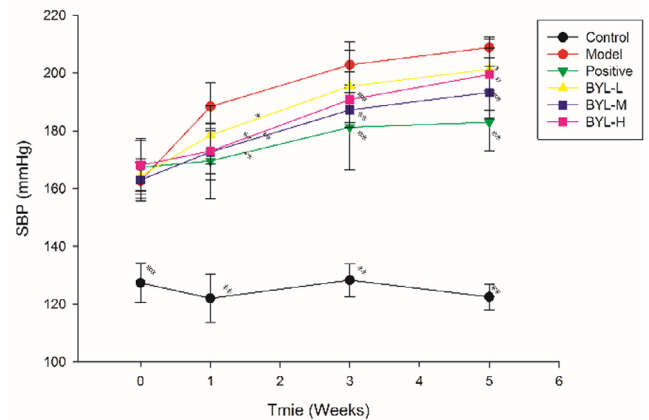
Fig. 1. Changes of systolic pressure during administration. $^*p < .05$, $^{**}p < .01$ versus the control group.

Table 3
BYL treatment affected selected serum indexes.

Group	Ang II (ng/mL)	ET-1 (ng/mL)	TGF-β (pg/mL)
Control	142.490 ± 11.233**	44.044 ± 14.505**	94.909 ± 6.929**
Model	276.867 ± 29.588$^{\#\#}$	99.056 ± 28.587$^{\#\#}$	189.790 ± 17.391$^{\#\#}$
Positive	162.538 ± 19.198$^{**,\#\#}$	54.249 ± 13.892**	122.122 ± 14.437**
BYL-L	234.540 ± 21.994$^{**,\#\#}$	79.430 ± 21.018$^{*,\#\#}$	162.497 ± 19.778$^{\#\#}$
BYL-M	201.531 ± 20.145$^{**,\#\#}$	73.427 ± 11.199$^{**,\#\#}$	119.802 ± 8.19**
BYL-H	167.235 ± 12.707$^{**,\#\#}$	62.897 ± 15.973$^{**,\#}$	96.273 ± 8.965**

Values are expressed as means ± SDs (n = 10). $^*p < .05$, $^{**}p < .01$ verus the Model group; $^{\#}p < .05$, $^{\#\#}p < .01$ verus the Control group.

myocardial fibers were slightly wavy (1 in Fig. 2B), the regional myocardial interstitial space was mildly extended (2 in Fig. 2B), and a small amount of loose connective tissue hyperplasia was observed outside the vessel (3 in Fig. 2B). For the positive control group, myocardial fibers were closely arranged, except for partial capillary telangiectasia (1 in Fig. 2C). Compared with rats in the model group, BYL treatment effectively alleviated the symptoms observed in cardiac muscles. For the BYL-L group, myocardial fibers were closely arranged, except for mild capillary telangiectasia and congestion (1 in Fig. 2D), and a few small round vacuoles were observed in the myocardium (2 in Fig. 2D). Cardiac cells from the BYL-M and BYL-H groups performed better than those in the BYL-L group; myocardial fibers were abundant, and the arrangement of the fibers was close without other obvious abnormalities (Fig. 2E and F).

3.4. Masson staining

Masson staining was used to diagnose myocardial fibrosis. Collagen fibers in the model group were mainly distributed in the myocardial fiber gap, and the myocardial interstitial space increased at the location of fibrosis, with disordered arrangements of collagen fibers (Fig. 3B). Collagen fibers in other groups were mainly distributed in the myocardial fiber gap, but showed no obvious influence on the arrangement of myocardial fibers (Fig. 3C–F). Quantification of the percent area of collagen fibers revealed significantly increased collagen fiber ratios in the myocardium of the model group compared with that of the control group (control group: 2.161% ± 0.864%; model group: 14.141% ± 4.398%; $p < .01$; Fig. 3G). Compared with that in the model group, the collagen fiber ratio in the myocardium significantly decreased
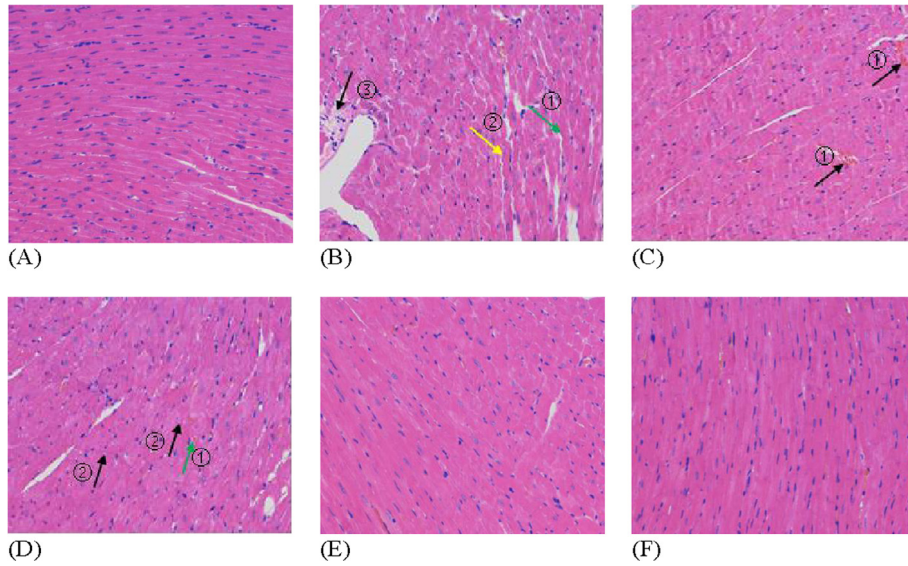
Fig. 2. Effects of BYL on myocardial morphology in rats (HE×200). (A) Control; (B) Model; (C) Positive; (D) BYL-L; (E) BYL-M; (F) BYL-H.
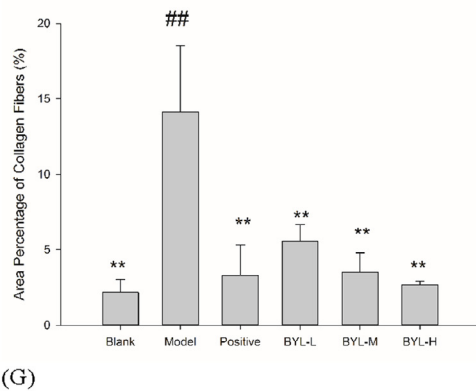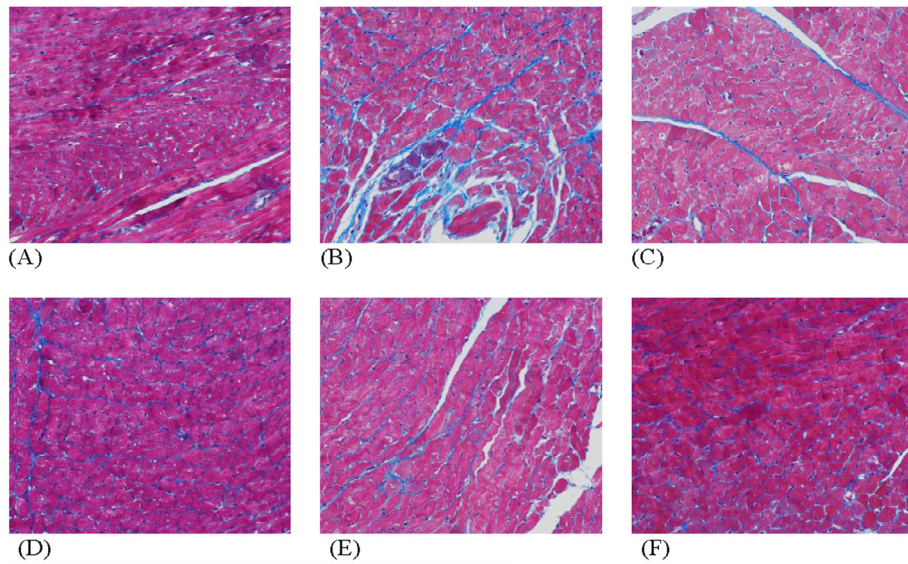
Fig. 3. Effects of BYL on myocardial collagen in rats (Masson staining, 200×). (A) Control; (B) Model; (C) Positive; (D) BYL-L; (E) BYL-M; (F) BYL-H. (G) The percent area of collagen fibers in myocardium (n = 3). The data are shown as means ± SDs. **$p < .01$ versus the blank group, ##$p < .01$ versus the model group.

following BYL treatment (BYL-L group: 5.544% ± 1.109%; BYL-M group: 3.486% ± 1.301%; and BYL-H group: 2.676% ± 0.228%; $p < .01$; Fig. 3G). The collagen fiber ratio in the myocardium of the positive control group significantly decreased compared with that of the model group (positive control group: 3.277% ± 2.021%, $p < .01$).

3.5. BYL treatment improved myocardial state via upregulation of PPAR-α and PPAR-γ

Real-time PCR showed that BYL treatment upregulated PPAR-α and PPAR-γ mRNA compared with that in the model group (Fig. 4). For PPAR-α, there was no significant difference between the model group and the BYL-L group ($p > .05$). However, PPAR-α mRNA expression in the BYL-M and BYL-H groups was significantly different from that in the model group ($p < .05$, $p < .01$, respectively). Like PPAR-α, PPAR-γ mRNA levels in the BYL-L group were not significantly different from that in the model group ($p > .05$), whereas those in the BYL-M and BYL-H groups were significantly different from that in the model group ($p < .05$).

4. Discussion

Many animal models have been used to study the pathogenesis of hypertension and the efficacy of antihypertension drugs (Pinto et al., 1998). Among these models, SHRs are popular in hypertension research (Tayebati et al., 2012). The disease process in SHRs is like that in human who have primary hypertension (Frohlich 1986, 1981). Moreover, organ damage, including cardiac hypertrophy, may occur in SHRs (Pinto et al., 1998). Therefore, we chose SHRs as the model animal to evaluate the effects of BYL on blood pressure and cardio protection.

In a previous study, the SBPs of SHRs began to increase during week 6 after birth and increased every week, reaching a peak at 25 weeks (Yao et al., 2003). Consistent with these findings, we demonstrated that the SBPs of SHRs increased throughout the study and that BYL treatment could control this increase in SBP, like the results observed in the positive control group. Elevated blood pressure can lead to an increase in terminal pressure, thereby increasing the burden on the heart. After cardiac overload, ventricular remodeling occurs. If the blood pressure can be controlled, damage to the corresponding target organ may be alleviated (Pinto et al., 1998). Moreover, greater interference with biochemical mechanisms may lead to improved protection of target organs (Pinto et al., 1998). Ang II plays an important role as a vasoconstrictor regulator in the renin-angiotensin system (Xiang et al., 2016) and can cause vascular smooth muscle cell proliferation and induce hypertrophy and fibrosis in myocardial cells after combining with corresponding receptors. Endothelin is currently the strongest vasoconstrictive peptide that regulates salt metabolism both in humans and in spontaneous hypertension animal models (Del Villar et al., 2005). ET-1 exerts its biological effects after binding with the corresponding receptor (Feldstein and Romero, 2007; Inoue et al., 1989) and is secreted from myocardial cells and fibroblasts (Suzuki et al., 1993). The presence of Ang II stimulates the secretion of ET-1 (Ito et al., 1993). TGF-β is an important molecule in the cardiovascular system (Sun et al., 2016); inhibition of TGF-β regulates fibrotic remodeling and reduces left ventricular remodeling (Kuwahara 2002; Villar et al., 2013). Recently, a study showed that ET-1 could not only enhance the epithelial-to-mesenchymal transition but also potentiate the TGF-β1-mediated acquisition of a mesenchymal cell phenotype (Wermuth et al., 2016). Interestingly, in our study, all BYL treatment groups showed reduced serum levels of Ang II, ET-1, and TGF-β, indicating that BYL treatment could reduce the secretion of ET-1 and modulate its related factors.

Effective treatments must reduce blood pressure and minimize myocardial remodeling-related damage, including myocardial fibrosis and myocardial hypertrophy. According to the pathological staining results, BYL treatment ameliorated myocardial damage in a dose-dependent manner in SHRs. Considering the changes in energy metabolism and the myocardial structure during myocardial remodeling, we chose PPAR-α and PPAR-γ to determine whether the drug influenced myocardial energy metabolism. PPARs, including PPAR-α and PPAR-γ, are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors (Toral et al., 2017). PPAR-α is responsible for lipid oxidation and is mainly expressed in the liver, kidney, and muscle (Desvergne and Wahli, 1999), whereas PPAR-γ is associated with adipocyte differentiation, lipid storage, and insulin sensitivity and is highly expressed in adipose tissue (Peter et al., 1994). Both proteins are expressed in the cardiovascular system (Duan et al., 2009). Activation of PPAR-α could inhibit ET-1 production and reduce interstitial and perivascular cardiac fibrosis (Ogata et al., 2002), whereas PPAR-γ activators regulate cardiomyocyte hypertrophy (Yamamoto et al., 2001). In the present study, BYL treatment, particularly BYL-M and BYL-H, upregulated the expression of PPAR-α and PPAR-γ transcripts in SHRs. Thus, these findings, combined with the observation that BYL improved the myocardial structure, suggested that the protective effects of BYL on the rat myocardium may be achieved through PPAR-α and PPAR-γ regulation.

Because of the natural origin of bee products, consumers may prefer to use bee products as dietary supplements rather than other products; thus, bee products have become increasingly prevalent worldwide. Flavonoids and flavonoid compounds have been reported to have vasodilatory and antihypertensive effects (Grassi et al., 2009; Parkar et al., 2016; Toh et al., 2013). Because of the abundance of flavonoids in propolis, this component has received much attention from researchers in the field of cardiovascular biology. Additionally, royal jelly is rich in vitamins, proteins, peptides, and fatty acids (Ramadan and Al-Ghamdi, 2012; Alrowais and Alyousefi, 2017), and some peptides in royal jelly inhibit angiotensin I-converting enzyme activity, resulting in antihypertensive effects in SHRs (Tokunaga et al., 2004). Melittin, apamin,
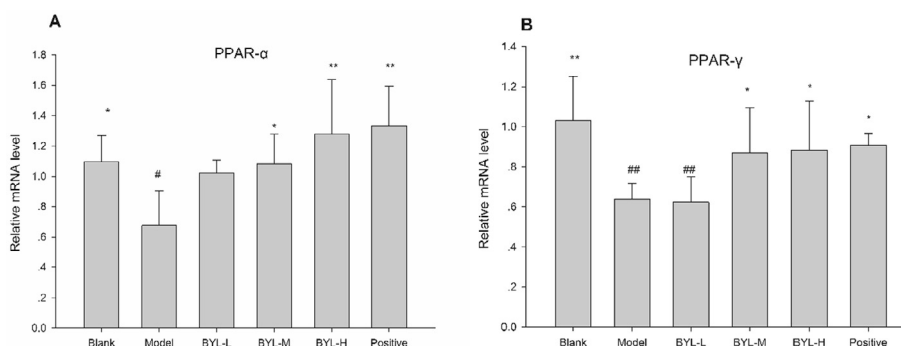

Fig. 4. Effects of BYL treatment of relative PPAR-α and PPAR-γ mRNA levels. $^*p < .05$, $^{**}p < .01$ versus the control. $^\#p < .05$, $^{\#\#}p < .01$ versus the blank.

and phospholipase A2 are the main components of bee venom. The individual components, as well as whole bee venom, have protective effects on the myocardium through regulation of calcium ions, magnesium ions, and inflammation (Kang et al., 2008; Saleh and Saleh, 2011; Wooram et al., 2010). Thus, many apitherapy products may have applications as medicines as well. In traditional Chinese medicine, the "sovereign, minister, assistant, and guide" rule (Wu et al., 2012; Gao et al., 2017) would support combining individual bee products having antihypertensive effects to achieve improved effects. However, further studies are needed to compare the effects of multi-component supplements with those of single ingredients to determine whether the observed effects are synergistic or are caused by certain compounds. Moreover, our findings showed that blood pressure was not dose dependent, suggesting that the differences may be related to individual variation. Thus, because this was an exploratory study in a rat model, the findings may be more conclusive if supported by other models or in vitro studies.

Acknowledgements

Author contributions

Xiaoqing Miao designed the experiment and provided the compound preparation. Yanru Sun conducted all experiments and analyzed data. Mingfeng Han helped with and supported the PCR assays and data analysis. Zhenhuang Shen helped with daily management of the animals. Haibo Huang helped with processing of animal samples.

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