Cardiovascular Protective Effects of Traditional Chinese Medicine Bak Foong Pills in Spontaneously Hypertensive Rats

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The cardiovascular protective effects of the traditional Chinese medicine Bak Foong Pills (BFP) were investigated. Spontaneously hypertensive rats (SHR) were treated (3 g/kg) over a 5-month period and blood pressure measurements periodically tested with a plethysmographic tail cuff. Following treatment, blood samples were analysed for serum electrolyte levels and lipid levels and brain tissue subjected to micro-array analysis. In vitro experiments were also conducted to identify possible direct vasorelaxatory effect. The results showed that BFP was able to significantly reduce both systolic and diastolic blood pressure by about 30 mmHg in SHR following 5 months of treatment, when compared to untreated animals. Investigation for possible mechanisms of actions revealed that BFP treated rats had elevated blood serum K⁺ levels, and also demonstrated decreased serum triglyceride levels. Micro-array analysis of brain tissue showed altered expression of acetylcholine and lysosphinogolipid receptor genes that are known to regulate blood pressure. In vitro experiments also showed that BFP caused a concentration-dependant vasorelaxation of isolated rat aortae when contracted with phenylephrine, which was partially inhibited by nitric oxide synthase inhibitor L-NAME (100 μm). These data suggest that BFP is able to significantly reduce hypertension in SHR through mechanisms probably involving a combination of increased serum K⁺, vasorelaxatory action, reduced serum triglyceride and altered gene regulation in the higher centres.

Key words Bak Foong Pill; anti-hypertensive; cardio-protective; estrogen

The incidence of hypertension and cardiovascular disease among women is low when compared to men of a similar age, but steadily increases after the onset of menopause, probably due to the loss of endogenous estrogen and thus the loss of its cardiovascular protective effects.¹ These protective mechanisms include direct and rapid vasodilatory effects on vascular smooth muscle via either a nitric oxide dependent pathway or, to a lesser extent, via stimulated opening of calcium-activated potassium channels in vascular smooth muscle, which results in lowered blood pressure. Other mechanisms involve longer-term genomic effects on vasodilatory enzymes, reduced vascular injury and increased vascular endothelial growth as well as indirect effects such as reduced serum triglyceride and blood coagulation.² The exact mechanisms by which estrogen mediates these cardiovascular protective effects is still poorly understood, but is suspected to mainly involve the estrogen receptors ER-α and ER-β which are expressed in vascular, reproductive, liver, bone and brain tissues of both men and women.³,⁴ The possible use of alternative sources of estrogens, that may have different specificity at different estrogen receptors, is becoming increasingly popular, with the major focus on development of phytoestrogens from plants and herbal medicines.

One such herbal medicine which is believed to have estrogen-like activity is Bak Foong Pills (BFP) (China registration #Z980035), an over the counter traditional Chinese medicine, which has been used for the last three centuries in China and South East Asia to treat female associated disorders such as irregular menstruation, dysmenorrhoea and post-partum fatigue.⁵,⁶,⁷ It has long been suggested that BFP possesses estrogenic properties due to its effectiveness in treating gynaecological problems. Indeed, our previous studies have suggested that BFP causes upregulation of cystic fibrosis transmembrane conductance regulator (CFTR), anti-platelet and anti-coagulation activity,⁶,⁷ which are known to be regulated by estrogen.⁸

The present study aims to investigate the cardio-protective effect of BFP by examining its effect on the development of high blood pressure in Spontaneously Hypertensive Rats (SHR), since SHR have been shown to share many of the characteristics associated with human primary cardiovascular disease.⁹ The possible mechanisms involved were also investigated by in vivo and in vitro methods.

MATERIALS AND METHODS

Animals Five week-old male SHR rats were supplied by the Laboratory Animal Services Centre, The Chinese University of Hong Kong. All experiments were performed in accordance to institutional Animal Ethics Committee guidelines.

Chemicals and Solutions Bak Foong Pills were obtained from Eu Yan Sang (HK) Ltd. (Hong Kong, China), whereas indomethacin and phenylephrine were supplied by Sigma Chemical Company (St. Louis, MO, U.S.A.) and L-NAME from Tocris (Bristol, U.K.). Krebs–Henseleit solution composed of NaCl (118.4 mM), KCl (4.6 mM), MgSO₄·7H₂O (1.2 mM), CaCl₂·2H₂O (2.5 mM), KH₂PO₄ (1.2 mM), NaHCO₃ (26.2 mM) and glucose (11.7 mM) all of which were purchased from Merck KGaA (Darmstadt, Germany). Triglyceride and Cholesterol levels were determined using enzyme immuno assay kits supplied by WDO Biological Eng. Co. Ltd. (China).

Blood Pressure Measurements Five SHR were randomly assigned to control (vehicle, 10 ml/kg dH₂O, p.o.) and BFP (3 g/kg, p.o.) groups and dosed 6-d a week for 5 months. Systolic and Diastolic blood pressure measurements in conscious rats were taken prior to treatment using the indirect

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plethysmographic tail cuff method followed by further readings midway and at the end of the treatment period. At least 10 determinations were made every session on each rat and the mean of 6 readings within a 5—10 mmHg range was taken as the blood pressure. At the end of the treatment period, direct blood pressure readings were taken in unconscious rats by cannulation of the left carotid artery under ketamine (75 mg/kg i.p.) and xylazine (10 mg/kg i.p.) anesthesia, 36 h following the last treatment, in order to confirm the tail-cuff measurements accuracy and to provide mean arterial pressure readings. Blood serum samples were also taken and analysed for cholesterol and triglyceride levels (enzyme immunoassays, according to manufacturers instructions) and renal function (Pathlab HK Ltd.) including sodium, potassium, chloride, urea and creatinine.

Micro-array In order to determine possible higher centre control of blood pressure by BFP, micro-array analysis was performed on brain tissue of BFP treated SHR and compared with vehicle treated animals. All the array procedures were performed according to the manual of Atlas cDNA Expression Arrays (BD Bioscience Clontech, Palo Alto, CA, U.S.A.) with some modifications. Briefly, total RNA was extracted from each tissue sample with Atlas Pure Total RNA Labeling System (BD Bioscience Clontech), and was treated with 1000 units of DNase I at 37 °C for 30 min. The quality of extracted total RNA was checked by spectrosocopy. Labeled cDNA probe was synthesized by reverse-transcription primer mix, [α-32P] deoxyATP (10 μCi/μl; 3000 Ci/mmol, Amersham Bio-science, Piscataway, NJ, U.S.A.), and reverse transcriptase at 50 °C for 60 min Atlas Rat 1.2 Array nylon membranes (BD Bioscience Clontech) were prehybridized with salmon sperm DNA in UltraHyb buffer (BD Bioscience Clontech) at 68 °C for at least 1 h. The nylon arrays were then hybridized at 68 °C overnight with the 32P-labeled cDNA probe (5—10×106 cpm) purified with Atlas Nucleospin Extraction Kit (BD Bioscience Clontech) to remove unincorporated radioisotope. After hybridization, the membranes were washed with solution 1 (2× saline sodium citrate (SSC; 0.3 M NaCl/0.03 M sodium citrate), 1% sodium dodecyl sulfate (SDS)) and solution 2 (0.1× SSC, 0.5% SDS) at 68 °C separately, and then washed with solution 2 at room temperature for 5 min. The hybridized membranes were exposed to a Cyclone Storage Phosphor Screen (PerkinElmer Life Sciences Inc., Boston, U.S.A.) for 1—2 d, and the radio-active signals were visualized with a Packard Storage Phosphor System (PerkinElmer Life Sciences Inc.). Array images were analyzed with Atlas Image software (BD Bioscience Clontech). Signal intensities of each gene were calculated by subtracting the background value from the density measurement of each gene. Local background was used for the calculation except in case of genes with high signal intensity above 10000 in both paired membranes when an external background measurement was applied. The genes whose adjusted signal intensities were below 0.5× the mean background value were removed from analysis. A normalization factor was determined by averaging the signal intensities of two house-keeping genes that were determined not to change from membrane to membrane.

Vascular Smooth Muscle Preparation Direct vasorelaxatory effect of BFP was measured using thoracic aortic ring preparations from adult male Sprague Dawley rats. Rings (3 mm) were mounted in organ baths and immersed in gassed (95% O2, 5% CO2) Krebs Henseleit solution containing indomethacin (3 μM), to prevent endogenous prostacyclin production, at a resting tension of 1 g. Tissue was primed with KCl (40 mM) followed by contraction with phenylephrine (100 nM) and cumulative addition of BFP (100 μg/ml—3 mg/ml). Nitric oxide-dependent relaxation was assessed by incubating L-NAME (100 μM) for 30 min prior to phenylephrine contraction.

Data Analysis Results were expressed as mean±S.E.M. unless otherwise stated. Statistical levels of significance (p<0.05) were determined using, unpaired t-test, one-way analysis of variance (ANOVA) or two-way ANOVA where appropriate. Non-linear regression was used to determine EC50 values (GraphPad Prism, V 3.03). Mean arterial pressure from direct carotid artery cannulation was calculated using the following formula: MAP=diastolic pressure+1/3 pulse pressure.

RESULTS

Blood Pressure Measurement Treatment with BFP caused reduction in both the development of hypertension and significantly reduced blood pressure in SHR. Untreated animals continued to develop hypertension and showed an increase of about 6 mmHg in systolic, and 10 mmHg in diastolic pressure from the start of treatment, whereas BFP treated animals showed reductions of about 36 and 29 mmHg respectively, whereas animals treated with BFP demonstrated reduced systolic and diastolic blood pressure of 156±5 and 78±4 mmHg respectively, whereas animals treated with BFP had systolic and diastolic hypertension of 183±5 and 105±6 mmHg respectively, whereas animals treated with BFP had systolic and diastolic hypertension of 183±5 and 105±6 mmHg respectively, whereas animals treated with BFP had systolic and diastolic hypertension of 183±5 and 105±6 mmHg respectively. These results were confirmed using the traditional cannulation of the carotid artery, with reductions in mean arterial blood pressure from 165±3.3 to 150±3.1 mmHg following BFP treatment (p<0.01, n=5, unpaired t-test) (Fig. 2). At the end of the treatment both plethysmographic and carotid artery readings demonstrated that BFP was able to approximately reduce systolic pressure.
by 15%, diastolic pressure by 26% and mean arterial blood pressure by 10% when compared to control animals at the same time point.

**Blood Serum Analysis** Serum electrolyte level measurements showed an increase in potassium levels following BFP treatment when compared to control, whereas there was no change in other serum electrolyte levels (Fig. 3). Analysis of blood serum for cholesterol and triglyceride levels revealed that animals treated with BFP had significantly ($p < 0.05$, unpaired $t$-test) lower levels of serum triglyceride ($1.66 \pm 0.07$ mmol/ml, $n = 5$) than untreated animals ($2.11 \pm 0.10$ mmol/ml, $n = 5$), whereas no difference in cholesterol levels were found following treatment with BFP (data not shown).

**Micro-array** Micro-array analysis of brain tissue for changes in gene expression exhibited upregulated expression of lysosphingolipid receptor and $K^+$ channel rectifier (RB-IRK2) genes, whereas downregulation was observed in acetylcholine receptors, glucose transporter protein, ras-related protein m-ras and phospholipase C$\beta$1 genes when compared to vehicle treated animals.

**Vascular Relaxation** Isolated organ bath preparations, used to investigate the *in vitro* effect of BFP, demonstrated that BFP was able to mediate a concentration dependent relaxation of rat aortae contracted with phenylephrine (EC$_{50}$ =
374±19 μg/ml, n=4). This response was significantly inhibited with the addition of the nitric oxide inhibitor L-NAME (100 μM) causing a rightward shift in the response (EC\textsubscript{50} = 1.6±0.07 mg/ml, n=4, p<0.001, Two-way ANOVA) (Fig. 5).

**DISCUSSION**

This study demonstrates that a single daily dose of BFP over a period of 5 months significantly reduces blood pressure in SHR, a rat model of hypertension. The untreated rats continued to develop further hypertension to the high pressure levels seen in other studies on SHR, where treated animals not only demonstrated arrest of the development of hypertension but also a reduction in blood pressure. The direct blood pressure measurements with carotid artery cannulation at the end of the experiment confirmed a significant reduction in mean arterial blood pressure in BFP treated rats. However, the blood pressure readings following carotid artery cannulation were somewhat higher than those taken by tail cuff. This was probably due to a combination of factors, firstly, indirect methods of blood pressure readings such as tail cuff do not give absolute levels of blood pressure and are only able to give approximate values in relation to the control treated group. Secondly, during direct measurement, anesthetics such as ketamine, can considerably raise blood pressure by as much as 40 mmHg thus giving the appearance of somewhat high mean arterial pressures. Despite the disadvantages of both methods, overall when compared to their appropriate control treated animals, both measurement methods show a reduction of between 26 and 10% in blood pressure following BFP treatment, thus confirming its anti-hypertensive effect.

The mechanism by which BFP is able to reduce blood pressure is likely to be a combination of a number of beneficial factors observed. Renal function test on rat serum demonstrated a slightly increased serum potassium level in BFP treated animals. It has long been known that elevated serum potassium levels, usually obtained by the use of potassium supplements or potassium sparing diuretics, can be useful in controlling hypertension due to increased renal output, and that estrogen treatment can increase the opening of calcium-activated potassium channels. \(^\text{1,14}\) Although increased serum potassium levels could cause hyperkalemia, the increased potassium levels observed were still within normal ranges with less than 30% change between control and BFP treated animals. \(^\text{15}\) Blood serum analysis also demonstrated significantly lower triglyceride levels in SHR treated with BFP when compared to untreated animals. Although reduction in triglyceride levels may not cause a direct reduction in hypertension, it is associated with reduced risk of cardiovascular disease due to reduced hardening of vascular tissue. \(^\text{16}\)

Micro-array analysis of brain tissue also indicated that BFP might be able to prevent development of hypertension by altering regulating mechanisms in the brain. The down-regulation of acetylcholine receptors (gamma and delta) in BFP treated animals may provide a clue to the anti-hypertensive mechanisms of BFP. Direct injection into the hypothalamus of cholinergic neurotoxins has been shown to cause significant reduction in blood pressure in SHR. \(^\text{17}\) Increased expression of lysosphingolipid receptor was also observed in BFP treated SHR when compared to control. Lysosphingolipids levels in the kidneys have been linked to the promotion of diuresis with increased blood K\textsuperscript{+} levels. \(^\text{18}\) Although there is no direct evidence of brain lysosphingolipid receptors being responsible for control of blood pressure, the ability of BFP to modulate expression of these receptors may provide an insight into its mechanism of action. The elucidation of the exact role of these and other genes, which had altered expression following BFP treatment, awaits further investigation.

In vitro experimentation demonstrated that BFP has a direct vasorelaxatory effect on rat aortae stimulated with phenylephrine. This is partly dependent on nitric oxide, since the nitric oxide synthase inhibitor L-NAME significantly inhibited the vasorelaxatory effect of BFP. It is therefore possible that some of the anti-hypertensive effect of BFP may be due to the direct vasorelaxatory effect of BFP.

Although the active ingredient(s) responsible for these observed effects are still unknown, the circumstantial evidence thus far suggest an estrogen-like actions may be at least partly responsible. Anti-hypertensive and cardio-protective effects mediated by estrogen are very similar to those mediated by BFP, such as, nitric-oxide mediated vasodilatation, anti-platelet, anti-coagulation and reduced triglyceride levels. \(^\text{1,13,19}\) Traditionally, the effects of BFP have been largely attributed to a supposed estrogenic effect, since increased estrogen levels have beneficial effects on many of these diseases traditionally treated by BFP. \(^\text{6}\) Although our previous studies have demonstrated that BFP is unlikely to have a direct estrogenic, it was shown to share some estrogen-like activities. \(^\text{6,5}\)

In conclusion, our study shows that BFP is able to significantly reduce blood pressure in SHR. The mechanism of action though not fully understood, involves elevated serum potassium levels, vasorelaxation, and the reduction in other cardiovascular risk factors such as serum triglyceride and the previously observed reduction in platelet aggregation and blood coagulation. \(^\text{7}\) The reduced acetylcholine receptor expression in the brain may also contribute to reduced development of hypertension in the SHR and the ability of BFP to regulate expression of various genes provides a direction for our future studies. The long history of use, with no reported side effects, together with the number beneficial effects observed in the present study suggests that BFP could be used for treatment of hypertension, maybe at least mild conditions. Further studies on the active ingredients, and the search for the possible involvement of estrogen-like compounds should reveal its precise mechanisms with a goal of providing an alternative treatment to current anti-hypertensive drugs.

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