Effects of Bak Foong Pills and Menoease Pills on White Blood Cell Distribution in Old Age Female Rats

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This study examined the effects of Bak Foong Pills (BFP) and the new BFP-derived post-menopause formula, Menoease Pills (MBFP), on the distribution of peripheral white blood cells (WBC) between BFP/MBFP-treated and non-treated rats. Eighteen months old female SD rats were used to mimic post-menopausal and old age animal models. The percentage distribution of lymphocytes, monocytes and granulocytes were measured using flow cytometry with and without treatments of BFP or MBFP. Results showed that WBC distribution in old age rats were significantly different from that of adult rats, suggesting that as the animal aged, their WBC distributions were altered. Old age rats were observed to have much lower percentages of lymphocytes, but higher percentages of granulocytes when compared to the adult rats, indicating possible attenuated immunity. Following treatment with BFP or MBFP, WBC populations were found to be redistributed back into the ranges observed in adult animals. Furthermore, MBFP, was found to alter WBC distribution in a dose-dependent manner. When compared to estrogen (E2), a well documented regulator of immune function, results showed that MBFP was able to show significantly greater effects on WBC redistribution compared to E2. However, in ovariectomised (ovx) old age rats, neither MBFP nor E2 treated groups showed any changes in WBC redistribution. These results indicate that MBFP may share similarities to E2. Indeed, the effect of MBFP and E2 seems to require intact ovaries, which are believed to be necessary for the modulation of WBC distributions and immune functions. Overall, our findings suggest that BFP and MBFP may be able to regulate WBC population in old age female rats, and thus, indicate their potential role on improving the attenuated immunity evident in post-menopausal and elderly women.

Key words Bak Foong Pill (BFP); Menoease Pill (MBFP); post-menopause; white blood cell (WBC); estrogen (E2); flow cytometry

The immune system is a defense mechanism that protects the body from infectious pathogens, such as viruses, bacteria, fungi, protozoa and multicellular parasites found in the environment.1) Immune responses are mediated by a variety of cells that originates from the bone marrow and differentiates into specific cell types in primary lymphoid organs. Some cells can be differentiated into white blood cells (WBC), which can circulate into the bloodstream or migrate into specific tissues for homing. The major classes of WBC include: antibody producing B lymphocytes and cytokine-releasing and cytotoxic T lymphocytes, phagocytic monocytes and macrophages, heavily granulated neutrophils, eosinophils and basophils, and other auxiliary mast cells, dendritic cells and platelets. Together, these cells form a main part of the immune system of our body and respond to environmental assaults.2)

The immune system declines with age, an onset that can occur as early as when an individual reaches sexual maturity. The decline is due to changes in hormone levels3) and in the immune cells, where cell loss, shift in the proportion of sub-populations, and qualitative cellular changes have all been detected.4—6) Age related immune decline has also been observed in the gastrointestinal-associated mucosa of humans. Older subjects exhibited lower levels of IL-2 secretion and IgA antibody production in response to stimuli, and showed slower cell proliferation and differentiation in peripheral blood mononuclear cells and lamina propria lymphocytes than those of younger subjects.7,8) Moreover, individuals with less efficient immune systems have been observed to have greater chance to bacterial infections.9,10) Although the links between sex hormones, such as estrogen (E2), and immune status in aging has not been firmly established; there is evidence showing a correlation between reduced hormone levels in post-menopausal women associated with increased risk of pathological and malignant diseases, such as higher blood pressure and cholesterol, osteoporosis, cardiovascular diseases, endometrial cancers and Alzheimer's diseases.9—15) Recent findings from women receiving hormone replacement therapy (HRT) suggest preservation or improvement of immune function associated with HRT by regulating the release of cytokines and cytokine secreting cells.16,17) In pre-menopausal women, there were high numbers of cytokine-releasing cells, whereas in post-menopausal women, there was a reduction in number of cytokines-secreting cells. Women receiving HRT, on the other hand, produced higher lymphocyte proliferation than untreated controls and showed slower signs to the development of post-menopausal symptoms.11,16,17) These findings suggest that HRT with E2 prevents post-menopausal women from an aberration of immune system by regulating cytokine genes and growth hormones and improving the balance of T-lymphocytes immune reactions.12,18,19)

Bak Foong Pills (BFP, also known as Bai Feng Wan), is a traditional Chinese medicine composed of 26 herbal ingredients (see ref. 20 for full list). BFP has long been used as a remedy to treat gynaecological disorders, such as dysmenorrhoea, irregular menstrual cycle and irregular bleeding. Clinical indications have suggested that BFP has beneficial effects on overall body function and our previous studies have demonstrated effects of BFP, most of which to various extents are estrogen-related, on improving blood circulation, gastrointestinal secretion21) and reduce the chance of cardiovascular hypertension.22) Menoease Pills (MBFP), is a new commercially available BFP-derived formula, with major ingredients including:
Radix Angelicae Sinensis, Rhizoma Chuanxiong, Radix Ginseng, to specifically attenuate post-menopausal symptoms. MBFP has been shown to function similarly to BFP to improve hormonal deviation commonly observed in post-menopausal and elderly females.

In the light of our previous findings with BFP and with our recent data showing a shift in WBC subpopulation in old age animals, we would like to investigate the effects of BFP and MBFP on WBC distribution in peripheral blood of old age rats compared to rats receiving no treatments. It is believed that BFP and MBFP may be able to exert a biological effect on regulating WBC proliferation and distribution that may be beneficial to the individual, however, the mechanism of actions are still unknown. It is suspected that BFP and MBFP may be able to exert a biological effect on WBC. In this study, we compare the new formula MBFP with E2 in ovari-intact or ovariectomised (ovx) old age rats to examine whether MBFP function in a similar manner to E2.

**MATERIALS AND METHODS**

**Materials** BFP and MBFP were obtained from Eu Yan Sang (Hong Kong) Limited. 17-β-Estradiol (E2) was from Sigma Aldrich, St. Louis, MO, U.S.A. Ketamine (10%) and xylazine (2%) anaesthetic were from Alfasan, Holland.

**Old Age Animal Model** Initially, female Sprague Dawley (SD) rats aging 22 to 24 months old were used as our old age animal model. However, study on these rats showed that many of them had developed tumours, which could affect our data analysis. As a result, we selected animals at the age of 18 months as our subsequent animal models. Rats aged 18 months have been demonstrated to be suitable model for postmenopausal-like and old age animals.17,21,22 Animals were supplied by the Laboratory Animal Service Centre of the Chinese University of Hong Kong. Animals were maintained in an air-conditioned room with controlled temperature of 24±2°C and humidity of 55±15%, in a 12 h light/dark cycle. The animals were given food and water ad libitum. Animals weighing between 300 g to 500 g were selected and randomly divided into control groups, BFP groups, MBFP groups and estrogen (E2) groups. There were on average 7 to 10 animals in each group. Experiments where ovariectomy was required, rats were ovariectomised at 9 months old and were aged to 18 months old before receiving treatment.

**Comparison of WBC Distribution between Adult and Old Age Rats** In order to compare if there were any differences in WBC distribution as the animal age naturally, we compared blood of 18 months old adult rats with 3 months old adult rats. Approximately 0.5 ml of blood was collected from each group (details of blood collection follows) and their lymphocytes, monocytes and granulocytes distributions were compared by flow cytometry.

**BFP Treatments in Adult and Old Age Rats** To observe the effect of BFP on WBC distribution in old age rats, 0.5 ml of blood was collected and their WBC distribution were compared against adult rats and against controls. Treatment rats were orally fed with water-dissolved BFP (3 g/kg/d) or vehicle (water, 10 ml/kg/d) for a period of 6 weeks. Following the treatment periods, another set of blood samples were collected and analysed by flow cytometry.

**Blood Collection** Blood was collected from each rat twice by heart-puncture method before and after treatment. Animals were firstly weighed and anaesthetized with ketamine (75 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) injection, and approximately 0.5 ml of blood was collected from their hearts directly using a 25G×5/8” gauge needle and quickly placed into 1.3 ml screw-cap microtube with EDTA (Sarstedt, Cat. No. 41.1395.005, Germany). Blood samples were gently mixed by hand at room temperature for 10 min. The rats were allowed to recover for 2 d before any treatments began.

**Flow Cytometry Analysis** Flow cytometer (Epics Altra, Beckman Coulter) was used to analyse WBC distribution. Collected blood samples were lysed for red blood cells (RBC) using Puragene® RBC lysis solution (Gentra Systems, Cat. No. D5001, Minneapolis, MN 55447, U.S.A.). One hundred microliters of blood sample was mixed with 130 μl of lysis buffer (ratio 1 to 1.3) and incubated at room temperature for 10 min. Approximately 4 ml of sheath buffer was added to dilute the blood concentration. WBC were analysed by cell size and granularity using the forward scatter (FS) and side scatter (SS) parameters, which were sufficient to distinguish the three subpopulation of WBC without the use of antibodies. A total cell number of 10000 cells were counted in each run by flow cytometer and cells falling within each gated subpopulations, as determined by FS and SS, were expressed as percentages (%) over the total number of cells counted.

**MBFP and E2 Treatment in Ovary-Intact and Ovariectomised Rats** In order to examine the associations between E2 and herbal medicine on immune functions, we compared E2 and the new formula MBFP on WBC distribution in ovari-intact or ovariectomised rats. Rats aged 9-months old were ovariectomised 9 months prior to our experiment. Ovary-Intact female SD rats were synchronized to identical cycles by hormonal injection (50 μg/kg, 17β-estradiol, i.p.) for 2 d. Blood was collected prior to the start of treatment with either MBFP (3 g/kg/d) or E2 (50 μg/kg/d, i.p. injected) for up to 6 weeks. Following the treatment period, further blood samples were collected and analysed by flow cytometry.

**Dose Dependent Effect of MBFP on WBC Distribution in Old Age Rats** Three different dosages of MBFP were tested in old age rats: low dose (1.5 g/kg/d), medium dose (3 g/kg/d) and high dose (6 g/kg/d) for 6 weeks to examine whether changes in WBC distribution are dosage dependent.

**Statistical Analysis** All results were expressed as mean±S.E.M and analysed using paired Student’s t-test or one-way analysis of variance (ANOVA), where appropriate. A “p” value <0.05 was considered to be statistically significant.

**RESULTS**

**WBC Distribution between Adult and Old Age Rats** Flow cytometry analysis showed there were differences observed in lymphocytes and granulocytes distributions between adult and old age female rats. WBC distribution for the 3 month old adult female rat was: lymphocytes (37±6.8%), monocytes (1.8±0.5%) and granulocytes (56±6.9%), which fall within the ranges described by Feldman for adult
rodents\textsuperscript{25}) (Fig. 1A). However, this WBC distribution pattern changed in old age rats with evident reduction in the levels of lymphocytes (11.1±2.7\%, \( p<0.01 \)), similar monocytes (1.0±0.2\%), but increased levels of granulocytes (84±4.4\%, \( p<0.01 \)) (Fig. 1B). This shift in WBC subpopulation pattern in old age rats had been previously observed and reported by Mohr.\textsuperscript{26}

Effects of BFP on Adults and Old Age Rats

There were significant changes to WBC distribution in old age animals treated with BFP, while no changes were observed for adult rats. BFP treatment on old age rats showed redistribution of WBC population from lymphocytes (20±5.7\%), monocytes (1.0±0.3\%), granulocytes (80±9.8\%) before BFP treatment to lymphocytes (43±4.5\%, \( p<0.01 \)), monocytes (1.6±0.2\%) and granulocytes (52±8.5\%, \( p<0.05 \)) after BFP treatment (Fig. 2B). No changes in blood profiles were observed in the water-treated control groups, confirming that the WBC distribution of untreated animals remained the same throughout the experimental period (Fig. 2C).

Effects of MBFP and E\textsubscript{2} on Ovary-Intact and Ovariec-
tomised Old Age Rats

In ovary-intact old age rats, the effect of MBFP showed significant changes in WBC distribution compared to E\textsubscript{2} treatment, where the effect of sex hormone on distribution of immune cells was less evident. MBFP showed that lymphocyte counts before and after treatments increased from 25±6.6\% to 46.6±2.7\% (\( p<0.05 \)), and granulocytes decreased from 65.6±8.8\% to 50.2±4.9\% (\( p<0.05 \)) (Fig. 3A). No significant changes were observed for monocytes. The effect of MBFP on WBC redistribution in old age rats also demonstrated a similar response to that observed with BFP. The effect of E\textsubscript{2} was less potent. E\textsubscript{2} increased lymphocytes from 17±5.4\% to 28.9±4.7\%, but a decrease in granulocytes from 81±8.8\% to 70±9.4\% (Fig. 3B). The effect of water as a control showed no significant changes before treatments (lymphocytes 20±6.4\%; monocytes 1.8±0.2\%; granulocytes 81.1±8\%) and after treatments (lymphocytes 24±4.2\%; monocytes 2.0±0.4\%; granulocytes 87.5±4.5\%) (Fig. 3C).

In ovariectomised old age rats, the effect of MBFP and E\textsubscript{2} showed little or no changes in WBC distribution. Ovx-MBFP rats showed WBC distribution before and after treatments: lymphocytes 15.3±9.9\% and 18.8±6.9\%, monocytes 0.9±0.5\% and 0.7±0.3\%, granulocytes 82.2±11.4\% and 88.1±4.6\% (Fig. 3D). Ovx-E\textsubscript{2} rats showed WBC distribution before and after treatments: lymphocytes 19.0±6.9\% and 20.2±4.2\%, monocytes 1.4±0.4\% and 1.0±0.1\%, granulocytes 79±7.3\% and 80.8±2.9\% (Fig. 3E).

Dose Dependent Effects of MBFP on Old Age Rats

Alterations in WBC redistribution in old age rats were observed in the treatments with MBFP. Changes were observed to be dosage responsive. At low dosage of MBFP (1.5 g/kg/d), WBC distribution were measured to be: lymphocytes (3.6±0.5\%), monocytes (1.5±0.1\%) and granulocytes (91.5±2\%), similar to the controls: lymphocytes (4.6±1\%), monocytes (1.8±1.4\%) and granulocytes (86.1±2\%). At medium dosage of MBFP (3 g/kg/d), WBC distribution...
showed significant differences to the control and were measured to be: lymphocytes (21.1±4.3%) \((p<0.05)\), monocytes (1.4±0.2%) and granulocytes (72.5±5%) \((p<0.05)\). At high dosage of MBFP (6 g/kg/d), WBC distribution showed greater significant differences to the control and were measured to be: lymphocytes (38.1±14.2%) \((p<0.05)\), monocytes (2.0±0.5%), granulocytes (48.3±12.1%) \((p<0.001)\) (Fig. 4).

**DISCUSSION**

The present study has demonstrated that BFP and MBFP were able to exert an effect on restoring the distribution of WBC in old age female rats. Many studies had demonstrated attenuated immunity in old age subjects correlated to the decline of T cell proliferation and B cell antibody production during aging, with increasing risks to infections observed in elderly people.\(^4\)\(^-\)\(^10\) The age-dependent pattern changes in WBC distribution can be observed in both humans and rodents, although the percentage changes in each species differ slightly. Studies conducted by Feldman\(^{25}\) and Mohr\(^{26}\) on rats...
indicated that the percentage range of WBC expected in adult rats were to be: lymphocytes (30—40%), monocytes (1—2%) and granulocytes (50—60%). However, during the aging process, the levels of lymphocytes deviate tremendously, while the levels of granulocytes increased. This transitional shift in WBC pattern is also observed to be similar in humans.\textsuperscript{4—8) The fact that under present experimental conditions, we observed similar percentages of immune cell subpopulations as previously reported in adult rats and the pattern of alteration in aged rats suggests that flow cytometry method was used appropriately.

Our findings illustrated a significant effect of BFP and MBFP in allowing WBC to redistribute from the old age range back into values within adult population meaning that these herbal remedies may be beneficial for improving WBC distribution in aged subjects. Although this observed difference in percentage of cell population could be due to redistribution of cell type, it is possible that a significant drop in granulocytes could also give the appearance of redistribution, in which case, a cell count in absolute numbers would provide a more solid support. However, this possibility is unlikely to be the case, since flow cytometry results in adult and ovx rats did not show any reductions in granulocytes, indicating that there were no toxic effects of the herbal remedies that could cause a sudden reduction in granulocytes. Hence, the observed WBC alterations in our results were true reflections in WBC distribution.

The effects of BFP/MBFP were found to be dependent on ovaries and were mimicked by E\textsubscript{2}, suggesting that ovarian E\textsubscript{2} may play an important role on immune regulation in the body as well as in mediating the effect of BFP/MBFP. This is also consistent with the observed effect on preservation or improvement of immune function in women receiving HRT.\textsuperscript{16) However, the present study also noted that the effect of E\textsubscript{2} was less potent as compared to that BFP or MBFP, indicating that other mechanisms, such as involving the higher center of the brain may also be involved.

Other evidences showed the immune system is also known to be regulated by the central nervous system (CNS). Although results regarding the CNS are not stated in this present study, the influence of neurofactors like neurotransmitters on the production and activation of WBC can be found in other reports.\textsuperscript{27) It has long been suspected that the effects of BFP on the female reproductive tract are mediated through the hypothalamus-pituitary-ovary axis. BFP/MBFP may exert their indirect effect on WBC by acting on the CNS as well as the ovary. In fact, other studies have observed altered expression of a number of genes including substance P and prolactin that are known to play roles in WBC regulation, in the pituitary of rats treated with BFP or MBFP.\textsuperscript{28—32) Interestingly, in a recent study, we also observed a direct effect of BFP on the release of dopamine,\textsuperscript{33) a neurotransmitter also considered to be an immune regulator.\textsuperscript{34) Therefore, the ability of BFP/MBFP to influence WBC observed in the present study may be mediated by, apart from ovarian E\textsubscript{2}, the actions of a number of neural factors including substance P, prolactin and dopamine, although the detail mechanisms remained to be elucidated. It should be noted the CNS is also under the influence of ovarian hormones. Therefore, the removal of the ovaries in the rats not only reduced blood levels of ovarian hormones but also impaired the functions of the higher centre, resulting in total abolishment of the BFP/MBFP effect on WBC redistribution as seen in the present study. The fact that BFP is able to exert an effect on aging but not in adult rats suggests that this is not a simple stimulatory action, but rather, a modulation through well-balanced acts of the neuro-endocrine system.

In conclusion, our results suggested that BFP and MBFP are able to significantly redistribute WBC populations of aged rats, an effect mimicked by E\textsubscript{2}. The present study also suggested that BFP/MBFP might function through an indirect mechanism involving the brain and the ovaries. The ability of MBFP to regulate WBC, at a dosage range comparable to that recommended for humans (10—15 g/d) to treat post-menopausal symptoms, may be beneficial for improving the attenuated immune function observed in post-menopausal and elderly women. Indeed, our current study only focussed on the changes in WBC distribution in old age rats with or without the treatment of BFP and MBFP. In order to complete the understanding of BFP and MBFP on the immune system, further studies on changes in functions and pheno-

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig4.png}
\caption{Dose-Dependent Effect of MBFP on Distribution of Lymphocytes (A), Monocytes (B) and Granulocytes (C) in Old Age Rats $n=17$, *p<0.05, ***p<0.001, by one-way ANOVA.}
\end{figure}
types of WBC will be needed.

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REFERENCES