

Letter to the Editor



Health Effect of Forest Bathing Trip on Elderly Patients with Chronic Obstructive Pulmonary Disease*

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Forest bathing trip is a short, leisurely visit to forest. In this study we determined the health effects of forest bathing trip on elderly patients with chronic obstructive pulmonary disease (COPD). The patients were randomly divided into two groups. One group was sent to forest, and the other was sent to an urban area as control. Flow cytometry, ELISA, and profile of mood states (POMS) evaluation were performed. In the forest group, we found a significant decrease of perforin and granzyme B expressions, accompanied by decreased levels of pro-inflammatory cytokines and stress hormones. Meanwhile, the scores in the negative subscales of POMS decreased after forest bathing trip. These results indicate that forest bathing trip has health effect on elderly COPD patients by reducing inflammation and stress level.

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality throughout the world. Exposure to air pollutants is one of the risk factors in the genesis of COPD. Previous studies have observed significant association between hospital admissions for acute exacerbation of COPD and the increase of air pollutants^[1].

COPD is characterized by a chronic inflammation of central and peripheral airways with concomitant destruction of the lung parenchyma. During exacerbation of COPD, the intensity of the systemic inflammation increases. Nature killer (NK) cells, nature killer T (NKT) cells and CD8+ T-lymphocytes play a key role in the pathogenesis of COPD by stimulating the production of inflammatory cytokines and chemokines, such as interleukin (IL)-6, IL-8, and C-reactive protein (CRP) etc^[2]. In addition,

these cells can cause targeted cell death through perforin/granzyme granule exocytosis pathway. Previous studies have identified an increased number of granzyme B positive cells both in peripheral blood and in induced sputum of COPD patients^[3].

Forest bathing trip is a short, leisurely visit to forest and is regarded as being similar to natural aromatherapy. Recently more attention have been paid to forest bathing trip due to its health-promoting effects. Our previous studies showed that forest bathing trip had positive effects on mental health by decreasing stress hormone levels and pulse rate, and it had therapeutic effects on human hypertension^[4-5]. In the present study, we aimed to investigate the effect of forest bathing trip on physiological and psychological responses of COPD patients. We supposed that forest bathing trip might modulate the inflammation level of COPD patients and exert beneficial effects on their health. Our findings indicated that forest bathing trip had resulted in decreased levels of intracellular perforin and granzyme B, accompanied by decreased levels of pro-inflammatory cytokines and stress hormones, which suggested the positive effect of forest bathing trip for COPD patients.

This study enrolled 20 COPD patients from Hangzhou, who had been without the acute exacerbation for at least 6 weeks. COPD was diagnosed according to the criteria of Global Initiative for Chronic Obstructive Lung Disease (GOLD). The inclusion and exclusion criteria has been showed in Table S1. The study was approved by the ethics committee of Zhejiang Hospital. Signed informed consent was obtained from every study participants. The study was performed at two

doi: 10.3967/bes2016.026

*This work was supported by funds from the National Natural Science Foundation of China (31301139 & 31201040) as well as funds from Science Technology Department of Zhejiang Province (2012C24005 & 2014C33130); Health Bureau of Zhejiang Province (11-CX01 & 2013ZDA002); and Zhejiang Provincial Key Disciplinary Fields of Geriatrics Program.

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different sites (forest and city) from 6 to 9 August 2013 as described in our previous study^[5]. Briefly, on the day before the study, the blood samples were taken from the participants in the morning before breakfast in our hospital. The participants were then randomly divided into two groups and sent to the study sites respectively. The study schedule is shown in Figure S1. Blood samples were collected in each study site before breakfast in the morning on 9 August 2013, then the study ended.

Cytokines production was determined with commercial ELISA kits as described previously (Bioleaf Biotech)^[4-5]. Relative and absolute numbers of NK, NKT-like, and CD8+ T cells, as well as perforin and granzyme B expression in these cells were determined using flow cytometry as reported previously^[3]. Whole blood samples were first stained with CD3-ECD, CD8-PC5, CD56-PC7 for 20 min at room temperature (Beckman). The red cells were then lysed and the resultant white cell pellet was permeabilized using cell permeabilization reagents (MultiSciences Biotech). Then the cells were stained with either anti-granzyme B and anti-perforin antibodies or the isotype control antibodies for 20 min at room temperature (BD Pharmingen). After washing, the cells were resuspended in 1% paraformaldehyde and stored at 4 °C in the dark prior to the analysis with flow cytometry. As for flow cytometric analysis, the samples were analyzed using a FAC Calibur flow cytometer (Becton Dickinson Immunocytometry Systems). The following gates were used to distinguish the 3 populations of interest: CD8+ T cells (CD3+/CD8+), NK cells (CD56+/CD3-), NKT-like cells (CD56+/CD3+). All populations were also restricted to a lymphocyte gate based on forward versus side scatter. The perforin-positive or granzyme B-positive regions were set by using isotype-matched negative control samples, and the positive percentage for each gate was reported. To present the data we used proportions of cells. To assess the fluctuating active mood states of the participants, we used the standard version of the POMS test as reported previously^[4-5]. We assessed the participants' mood state changes before and after the study.

The data are expressed as mean±SEM. Statistical analysis was performed using SPSS version 17.0 (SPSS China). Between-group difference was evaluated using unpaired *t*-tests or Wilcoxon Mann-Whitney tests. Repeated measures were analyzed using paired *t*-tests. Chi-squared test was

used to compare proportions. A *P*-value less than 0.05 was considered statistical significant.

The clinical characteristics of the participants are shown in Table S1. There was no statistical difference between the two groups in terms of age, body mass index, blood pressure or pulse. The numbers of participants taking inhaled or oral corticosteroids were not significantly different between the two groups. Furthermore, there was no statistical difference in lung function indicators, including FEV₁ (%pred), FEV₁/FVC (%), modified Medical Research Council (mMRC) dyspnoea scale or COPD assessment test (CAT) scores. None of the participants reported any adverse reaction during the study period.

NK, NKT-like and CD8+ T-cells are the three main classes of human killer cells involved in the pathogenesis of COPD, which can cause targeted cell death through perforin/granzyme granule exocytosis pathway^[2]. In this study we analyzed the proportions of these cells and intracellular expression of perforin and granzyme B in the peripheral blood of the study participants. Representative flow analysis dot plots for identification of CD8+ T-lymphocytes (CD3+CD8+), NK cells (CD3-CD56+) and NKT-like cells (CD3+CD56+) are shown in Figure 1A. As shown in Figure 1B, in the forest group, there was no significant change in the proportions of lymphocyte subsets compared with their baseline value. Similarly, no significant change was observed in any lymphocyte subsets in the peripheral blood from the participants in the city group. However, we found that in the forest group, the proportions of CD8+ T-cells, NK cells and NKT-like cells expressing perforin significantly decreased after the forest bathing trip (Figure 1C). Besides, we also noticed a slight reduction of granzyme B expression in the CD8+ T-lymphocytes in the forest group. In the city group, although there was a reduction in perforin level, it had no significance (Figure 1C). The proportion of granzyme B positive NK cells or NKT-like cells did not change in either of the two groups before or after the study (Figure 1D). Perforin and granzyme B play a key role in the disease process of COPD. Moreover, perforin and granzyme-B have been shown to increase significantly in both selected bronchial epithelial cells and peripheral blood T-cell populations from COPD patients^[3]. These results suggested that the forest bathing trip improved the health of COPD patients by reducing the intracellular perforin level.

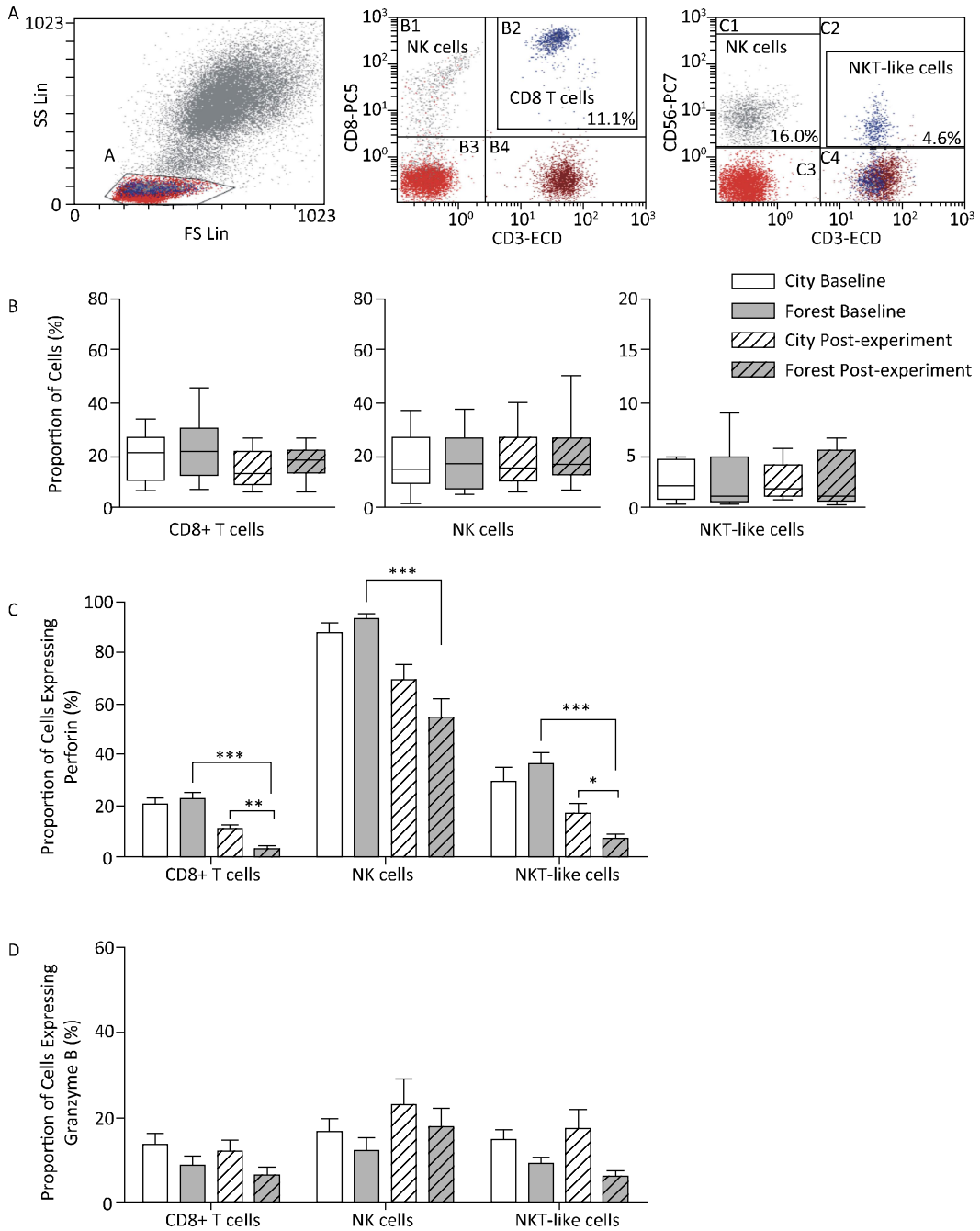


Figure 1. Effect of forest bathing trip on lymphocyte subsets in peripheral blood of COPD patients. A, Representative flow cytometric dot plots for identification of lymphocyte subsets in COPD patients. Percentages of cells within the quadrants are given; B, Proportion of NK, NKT-like, and CD8+ T-lymphocyte subsets. Data was expressed as a percentage of total lymphocytes; C, Proportion of NK, NKT-like and CD8+ T-lymphocytes expressing perforin; D, Proportion of NK, NKT-like, and CD8+ T-lymphocytes expressing granzyme B. (n=18) *: P<0.05; **: P<0.01; ***: P<0.001.

Systemic inflammation is also of great importance in the pathogenesis of COPD. The intensity of the systemic inflammation increases during the exacerbation of COPD^[6]. So we examined the serum levels of multiple pro-inflammatory factors. Our results showed that after the forest bathing trip, the serum levels of interferon- γ (IFN- γ), interleukin-6 (IL-6) and interleukin-8 (IL-8) decreased significantly in the forest group compared with their baseline values, which were consistent with our current findings for perforin (Figure 2). In addition, the levels of interleukin-1 β (IL-1 β), tumor necrosis factor α (TNF- α) and C-reactive protein (CRP) also decreased slightly after the forest bathing trip. However, no significant change was observed in the city group. IL-6, IL-1 β , and TNF- α are primary inflammatory cytokines which respond to the acute insult by orchestrating and releasing a cocktail of inflammatory mediators and protect the body from harm^[6]. In general, COPD patients have higher serum levels of IL-6, IL-1 β , TNF- α , and CRP than those without COPD. IL-8 and CRP levels increased in acute COPD exacerbation^[6]. In addition, the levels of IL-8, CRP, and TNF- α decreased significantly with corticosteroid treatment in patients with COPD^[7]. These results suggested that the forest bathing trip had effect to reduce the systemic inflammation level of COPD patients by downregulating the expression of pro-inflammatory cytokines.

To further investigate the mechanism of positive effect of forest bathing trip on COPD patients, we measured the expression of several COPD associated factors including pulmonary and activation-regulated chemokine (PARC/CCL-18), surfactant protein D (SP-D) and tissue inhibitor of metalloproteinase (TIMP-1). The elevated serum levels of these factors are associated with acute exacerbations of COPD^[8]. Moreover, the short-term use of oral corticosteroids can down-regulate systemic expression of these factors^[9]. In the present study, we observed a significant lower levels of PARC/CCL-18 ($P<0.01$) and TIMP-1 ($P<0.05$) in the forest group after forest bathing trip (Figure 3A). In addition, the decreased level of SP-D was observed in the forest group at the end of the study, although the change was not significant. However, no significant change of these factors was observed in the city group after the study. These results further support the hypothesis that forest bathing trip can improve the health of COPD patients.

Previous studies have demonstrated that forest bathing trip has positive effects on mental health of healthy adults, mainly by reducing the stress levels^[4]. In the present study, we also examined the psychological responses to forest bathing trip in COPD patients. POMS data indicated that forest bathing trip can decrease the intensity of the negative mood state such as tension, depression and

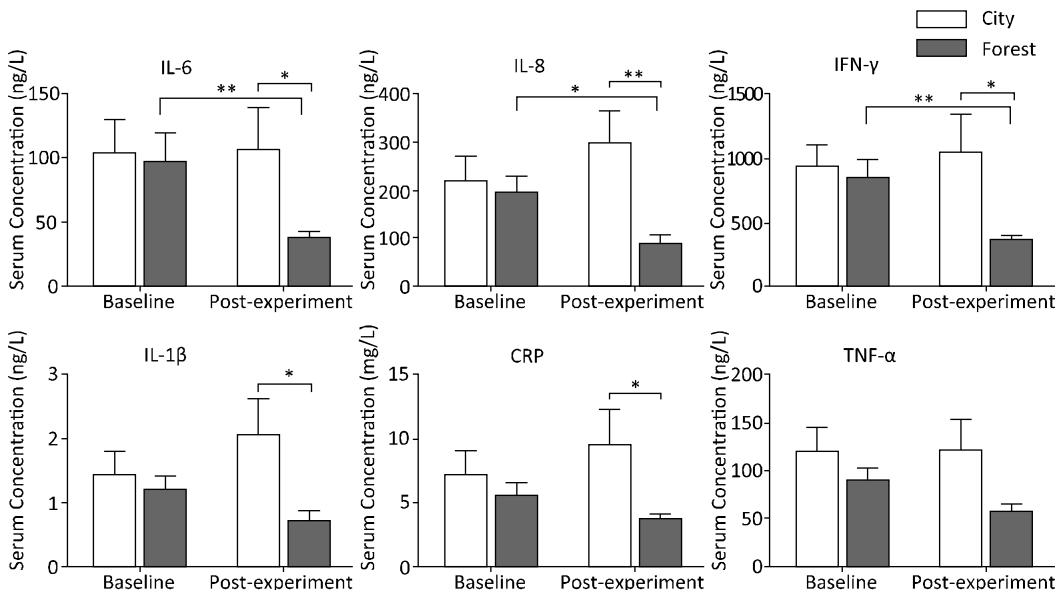


Figure 2. Effect of forest bathing trip on serum levels of pro-inflammatory cytokines. Interleukin-6 (IL-6), interleukin-8 (IL-8), interferon- γ (IFN- γ), interleukin-1 β (IL-1 β), tumor necrosis factor α (TNF- α) and C-reactive protein (CRP) of subjects were evaluated before and after the experiment. ($n=18$) * : $P<0.05$; ** : $P<0.01$.

anger, while city environment cannot (Figure 3C). The change in emotional states is important because it relates to the risk of COPD exacerbations and hospitalizations^[10]. Besides, we also detected the serum level of cortisol and epinephrine of the study participants. These two hormones are major components of the physiological stress response and are usually excreted in response to stress. Our results showed that both epinephrine level and cortisol level decreased in the forest group after forest bathing trip, while there was no change observed in the city group (Figure 3B). These results indicated that forest bathing trip could reduce the

stress level of COPD patients.

In conclusion, despite the small sample size, this study clearly showed that forest bathing trip resulted in significantly decreased levels of intracellular perforin and granzyme B, accompanied by the decreased levels of pro-inflammatory cytokines and stress hormones, which suggested the positive effect of forest bathing trip on elderly COPD patients. These initial positive results are encouraging and shows the necessity of randomized clinical trials with larger sample size and longer intervention in the future. It would be also interesting to investigate the duration of benefit of forest bathing trip in the future study.

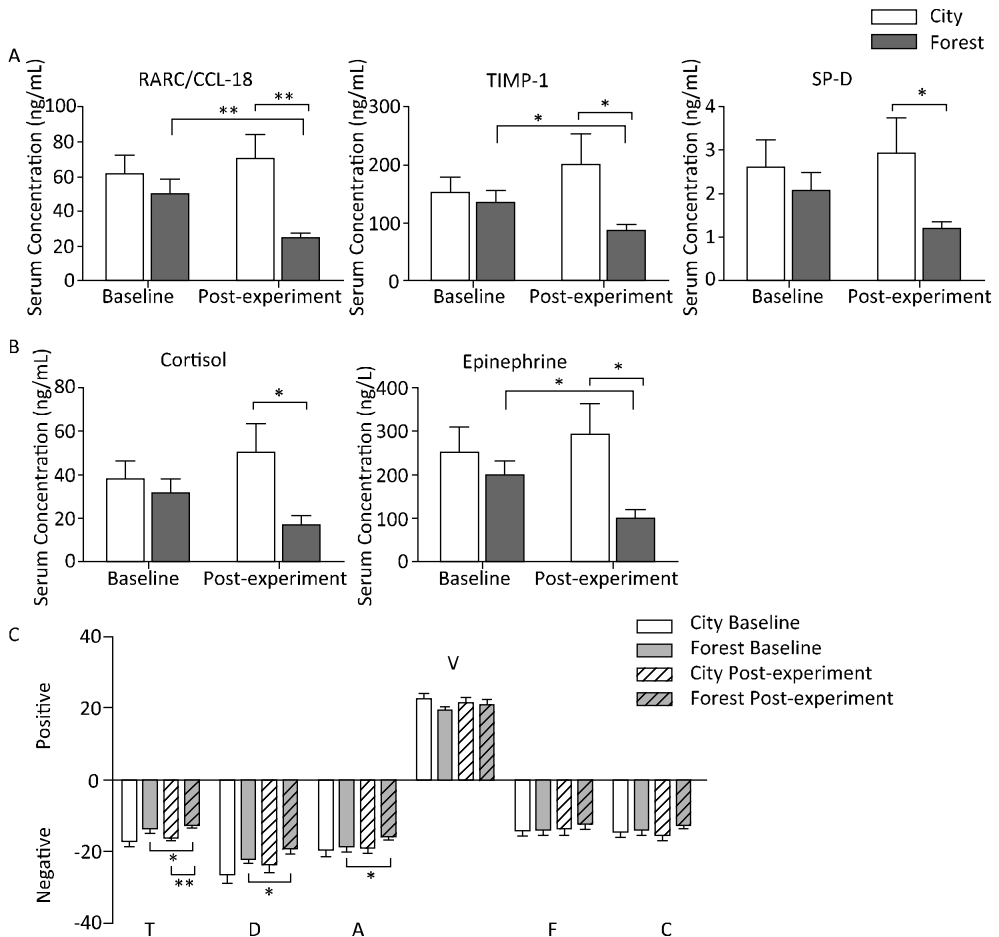


Figure 3. Effect of forest bathing trip on COPD-associated factors and stress levels of COPD patients. A, Pulmonary and activation-regulated chemokine (PARC/CCL-18), tissue inhibitor of metalloproteinase-1 (TIMP-1), surfactant protein D (SP-D). B, The levels of cortisol and epinephrine E of the participants were evaluated before and after the study. C, Profile of mood states (POMS) evaluation of the participants exposed to forest or city environment. The standard version of POMS including negative subscales (T: tension-anxiety; D: depression-dejection; A: anger-hostility; F: fatigue-inertia; and C: confusion-bewilderment) and a positive subscale (V: vigor-activity) was used to evaluate the participants' mood changes before and after the study. ($n=18$) * : $P<0.05$; ** : $P<0.01$.

ACKNOWLEDGEMENT

We would like to thank the patients who participated in this study for their time and cooperation. We appreciate the assistance of Ms. ZHOU Xiao Qiong (Associate chief technician in Department of clinical laboratory in Zhejiang Hospital, China.) for her helpful advice on flow cytometry analysis.

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Received: July 29, 2015;

Accepted: February 16, 2016

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Table S1. Clinical Characteristics of the Participants

Items	Forest	City	P Value
Participants	n=10	n=8	
Age (years)	70.1 (67-77)	70 (61-79)	0.570
Gender (male/female)	7/3	5/3	0.563 ^b
BMI (kg/m ²)	22.1 (18.3-25.7)	23.2 (17.9-28.1)	0.481
SBP (mmHg)	140.2 (105-154)	125.3 (104-139)	0.078 ^a
DBP (mmHg)	75.5 (64-89)	69.6 (57-80)	0.625 ^a
Pulse	79 (62-101)	75 (63-97)	0.475
Current smoking status (yes/no)	4/6	4/4	0.520 ^b
FEV ₁ (%pred)	63.13 (35-98)	66.35 (40-92)	0.152
FEV ₁ /FVC (%)	60.04 (31-70)	62.15 (37-69)	0.181 ^a
ICS (on/off)	2/8	2/6	0.618 ^b
LABA (on/off)	2/8	2/6	0.618 ^b
Cholinergic receptor antagonists (on/off)	0/8	1/6	0.467 ^b
mMRCdyspnoea scale	2.1 (1-4)	1.8 (1-3)	0.826 ^a
CAT score	15.5 (4-25)	14 (8-20)	0.614

Note. Results are expressed as median with range in brackets. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FEV₁, forced expiratory volume in 1 second; pred, predicted value; FVC, forced vital capacity; ICS, Inhaled corticoids; LABA, Long-acting β -agonist; mMRC, modified Medical Research Council questionnaire; CAT, COPD assessment test. ^aMann-Whitney test was used; ^bChi-squared test was used; other data were analyzed using the unpaired *t*-test.



Figure S1. The experimental protocol for subjects exposed to the forest or city environment.