A FOREST BATHING TRIP INCREASES HUMAN NATURAL KILLER ACTIVITY AND EXPRESSION OF ANTI-CANCER PROTEINS IN FEMALE SUBJECTS

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Received December 27, 2007 – Accepted January 13, 2008

We previously reported that forest bathing trips enhanced human NK activity, number of NK cells, and intracellular anti-cancer proteins in lymphocytes, and that the increased NK activity lasted for more than 7 days after the trip in male subjects. In the present study, we investigated the effect of forest bathing trip on human NK activity in female subjects. Thirteen healthy nurses, age 25-43 years, professional career 4-18 years, were selected with informed consent. The subjects experienced a three-day/two-night trip to forest fields. On day 1, the subjects walked for two hours in the afternoon in a forest field; on day 2, they walked for two hours each in the morning and afternoon in two different forest fields; and on day 3, the subjects finished the trip and returned to Tokyo after drawing blood and completing a questionnaire. Blood and urine were sampled on the second and third days during the trip, and on days 7 and 30 after the trip. NK activity, numbers of NK and T cells, and granulysin, perforin, and granzymes A/B-expressing lymphocytes in the blood samples, the concentrations of estradiol and progesterone in serum, and the concentrations of adrenaline and noradrenaline in urine were measured. Similar control measurements were made before the trip on a normal working day. The concentrations of phytoncides in the forests were measured. The forest bathing trip significantly increased NK activity and the numbers of NK, perforin, granulysin, and granzymes A/B-expressing cells and significantly decreased the percentage of T cells, and the concentrations of adrenaline and noradrenaline in urine. The increased NK activity lasted for more than 7 days after the trip. Phytoncides, such as alpha-pinene and beta-pinene were detected in forest air. These findings indicate that a forest bathing trip also increased NK activity, number of NK cells, and levels of intracellular anti-cancer proteins in female subjects, and that this effect lasted at least 7 days after the trip. Phytoncides released from trees and decreased stress hormone levels may partially contribute to the increased NK activity.

A forest bathing trip, which is similar to natural aromatherapy, involves a visit to a forest field for the purpose of relaxation and recreation by breathing in volatile substances, called phytoncides, consisting of antimicrobial volatile organic compounds derived from trees, such as alpha-pinene and limonene

Key words: anti-cancer proteins, female subjects, forest bathing, granulysin, granzyme, NK activity, perforin

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0393-974X (2008)

(1-3). Forest bathing trips are possible in similar environments throughout the world. We found previously that forest bathing trips increased human natural killer (NK) activity, NK cell numbers, and intracellular levels of perforin, granulysin (GRN), and granzymes A/B (GrA/B) in peripheral blood lymphocytes in male subjects (2-3) and that tree-derived phytoncides, such as alpha-pinene and limonene, enhance NK activity and intracellular levels of perforin, GRN, and granzyme A in NK cells *in vitro* (1). Komori et al (4) also reported that citrus fragrance found in forest affects the human endocrine and immune systems in depressed male inpatients as analyzed by the measurement of urinary cortisol and dopamine levels, NK activity, and CD4/8 ratios. Nevertheless, the question remained to be resolved whether forest bathing trips also increase NK activity in female subjects. In the present study, we addressed this question.

MATERIALS AND METHODS

Subjects

Thirteen healthy and non-pregnant female nurses, aged 25-43 years (mean 28.8±4.6), professional career 4-18 years (mean 6.7±3.8), were selected from Nippon Medical School hospital in Tokyo, Japan for the present study. Information gathered from a self-administered questionnaire, including age and lifestyle habits that asked about cigarette smoking, alcohol consumption, eating breakfast, sleeping hours, working hours, physical exercise, nutritional balance, and mental stress, have been reported previously (2-3, 5). Since the menstrual cycle influences NK activity (6), information on the menstrual cycle from all subjects was also gathered. Written informed consent was obtained from all subjects after a full explanation of the study procedures. None of the subjects had any signs or symptoms of infectious disease, used drugs that might affect immunological analysis, or were taking any medications at the time of the study. The Ethics Committee of the Nippon Medical School approved this study (approval No. 16-1).

Forest bathing trip

The subjects experienced a three-day/two-night trip to three different forest fields at Shinano town in Nagano prefecture located in northwestern Japan in early September, 2007. On the first day, the subjects walked for two hours in the afternoon in a forest field, and then stayed at a nearby hotel within the forest. On the second day, the subjects walked for 2 hours each in the morning and afternoon in two different forest fields, and then stayed at the same hotel. On day 3, the subjects finished the trip and returned to Tokyo after drawing blood and completing a questionnaire. Each walking course was 2.5 km, which closely resembled the normal physical activity for the subjects on an average working day. Daily physical activity of the subjects was monitored with a pedometer (2-3), and the level of background walking steps of the subjects on normal working days were monitored for a week, and the averaged walking distance of all the subjects was about 5.0 km/day. Then, we set the walking distances in the trip based on the result. The duration of sleep was measured with a piezo-electric accelerometer, Actiwatch (R) (Mini Mitter Co. Inc., Sunriver), worn on the wrist of the non-dominant arm (2-3, 7). Blood was sampled on the second and third days during the trip, and on days 7 and 30 after the trip, and three days prior to the trips as a control. Since it has been reported that human NK cell activity shows circadian rhythms (8-9), all samples were obtained at 8:00 am. All blood samples were placed in an ice/water box at 4°C and assays were performed within four hours from the blood being drawn. White blood cell (WBC) counts, NK activity, proportions of NK and T cells, and GRN, perforin, and granzymes A/B-expressing cells in peripheral blood lymphocytes (PBLs) were measured. Adrenaline and noradrenaline concentrations in urine were also determined. Since estradiol and progesterone affect human NK activity (10-13), estradiol and progesterone concentrations in serum were also determined.

NK activity

PBLs were separated from peripheral blood with a BD Vacutainer CPT (Becton Dickinson, Franklin Lakes, NJ, USA), and then adjusted to $4x10^6$ cells/ml for the assay of NK activity. NK activity was assayed according to a standard method (2-3, 14). Briefly, K-562 target cells were labeled with a sodium ^{51}Cr-chromate solution (Perkin Elmer, Boston, MA, USA) for 60 min at 37°C in 5% CO_2 and washed 4 times in RPMI-1640 containing 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA). The target cells were plated into round-bottomed 96-well microplates, then the effector cells (PBLs) at $4x10^6$, $2x10^6$, and $1x10^6$ cells/ml in 100 µl were added to the wells in triplicate at E:T ratios of 40:1, 20:1, and 10:1. Following a 4-h incubation at 37°C in 5% CO_2, the microplates were centrifuged and 100 µl of supernatant from each well was collected and measured in a gamma counter. Then, the NK activity was calculated as described previously (2-3, 14). For laboratory controls of the NK activity assay, we used the same K-562 cells as the target in all experiments and always kept the K-562 cells in the same conditions before the experiments, e.g.

we always used the K-562 cells 96-hr after thawing out the cells (3).

Cell staining and flow cytometric analysis

The surface markers of PBLs were stained with fluorescein isothiocynate (FITC)/phycoerythrin (PE) - CD16 and PerCP-Cy5.5-CD3 monoclonal antibodies (BD PharMingen, San Diego, CA, USA) for NK and T cells, and FITC/PE/PerCP-Cy5.5-mouse IgG1 as negative controls, for 30 min in the dark. Then, the cells were fixed/permeablized with Cytofix/cytoperm solution (BD PharMingen) for 20 min at 4°C, and then intracellular perforin and GrA/B were stained with FITC- anti-human perforin and FITC-GrA/B antibodies, respectively, with FITC-IgG2b for perforin and FITC-IgG1 for GrA/B as negative controls (BD PharMingen) for 30 min at 4°C according to the manufacturer's instructions. Intracellular GRN was stained with a rabbit anti-human GRN polyclonal antibody and rabbit serum as the negative control (1-3, 5) after fixation/permeablization with Cytofix/cytoperm solution, and then stained with FITC-goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA, USA) for 30 minutes at 4°C in the dark. After staining, the cells were washed twice with fixative solution and once with PBS containing 1% FBS. Flow cytometric analysis was performed with a FACScan flow cytometer as described previously (2-3, 5, 15). Lymphocytes were identified by their characteristic appearance on a dot plot of FSC versus SSC and electronically gated to exclude dead cells and granulocytes. The fluorescence gates were set using negative controls. For laboratory controls in the cell staining and flow cytometric analysis, we used antibodies from the same lot and we always added the same volume of antibodies to the cells with the same volume of buffer in all experiments, e.g. we added 10 µl FITC-anti-granzyme A into the cell suspension in 30 µl PBS in all experiments (3).

Urinary adrenaline and noradrenaline measurements

The levels of adrenaline and noradrenaline in urine were measured by an HPLC method using an HLC-725CAII analyzer. The instrument features a column-switching system composed of two pretreatment columns, one separation column, and a high-sensitivity detection unit based on a post-column reaction using a fluorogenic reagent, 1,2-diphenylethyleneamine. The detection limits of adrenaline and noradrenaline in urine were 8 and 14 fmol/ml, respectively (3, 16).

Measurements of estradiol and progesterone in serum

The levels of estradiol and progesterone in serum were measured by a chemiluminescent microparticle immunoassay (Architect estradiol and Architect progesterone, respectively, Abbott Japan, Tokyo, Japan). The detection limits of estradiol and progesterone in serum were 17.9 pg/ml and 0.1 ng/ml, respectively (17).

WBC count.

WBC, RBC, and platelet counts, the percentages of granulocytes, lymphocytes, and macrophages in peripheral blood, and the concentration of Hb, Hct, MCV, MCH, and HCHC were determined by an automatic cell counter (LC-550, Horiba Co., LTD. Kyoto, Japan) as described previously (2-3).

POMS test

The Profile of Mood States (POMS) test was used to examine mood changes of each subject before and after forest bathing using the POMS test in Japanese (2).

Measurements of phytoncides and environmental temperature/ humidity in the forest fields during the investigation

The concentration of volatile organic compounds (phytoncides), temperature, and humidity in the forests were measured as reported previously (2-3).

Statistical analysis

We analyzed the data using two-way ANOVA with no-repeated measures (one-way ANOVA with repeated measures), with the variability among individuals and the different days as two factors. Comparisons between different days were made with the paired t-test if the analysis of variance was significant. The analyses were performed with the Microsoft Excel software package for Windows. The significance level for p values was set at < 0.05.

RESULTS

Effect of a forest bathing trip on NK activity

The forest bathing trip significantly increased human NK activity, and this increase lasted for more than 7 days (Fig. 1).

Effect of a forest bathing trip on $CD16^+$ NK cells

The forest bathing trip significantly increased the percentage of $CD16^+$ NK cells, and this increase lasted for more than 7 days after the trip (Fig. 2). The forest bathing trip did not affect lymphocyte or WBC counts.

Effect of a forest bathing trip on the percentage of cells expressing cytolytic molecules

The forest bathing trip significantly increased the percentages of GRN, perforin, and GrA/B-expressing cells in PBLs, and this increase lasted for

more than 7 days after the trip (Fig 3).

Effect of a forest bathing trip on T ($CD3^+$) cells.

The forest bathing trip significantly decreased the percentage of T cells in almost all subjects after the trip compared with the control measurement. There were significant differences between before and after the trip on days 1, 2, and 7 in the percentage of T cells (Fig. 4).

Effect of a forest bathing trip on adrenaline and noradrenaline concentrations in urine

The forest bathing trip significantly decreased the concentrations of adrenaline and noradrenaline in urine (Fig. 5).

Changes of estradiol and progesterone concentrations in serum before, during, and after the trip

There was no significant change in the concentration of estradiol in serum before, during, or after the trip. The concentration of progesterone in serum 30 days after the trip was significantly higher than that before the trip. On the other hand, although the concentration of progesterone in serum on days 1 and 2 was higher than that before the trip, the difference was not significant (Fig. 6).

Effect of forest bathing trip on the score of POMS test

The forest bathing trip significantly increased the score for vigour and decreased the scores for anxiety, depression, anger, fatigue, and confusion in the POMS test (Fig. 7).

There were no significant differences in daily physical activity before and during the trip (data not shown). The hours of sleep increased during trip compared with control days (data not shown).

Lastly, phytoncides, such as alpha-pinene, beta-pinene, tricyclene, camphene, and isoprene, were detected in the forest fields during the investigation (Table I), but were not detected in the urban area of Tokyo. Weather during the forest bathing trip was excellent with average temperatures and humidity of 19.13 ± 0.23°C, 99.9 ± 0.00% on day 1 in the afternoon, 20.97 ± 0.12°C, 73.80 ± 2.74% on day 2 in the morning, and 18.97 ± 0.05°C, 90.87 ± 0.9% on day 2 in the afternoon in the forest fields during the walks. The average temperature and humidity in the urban area of Tokyo on the control day was 28.63°C and 59%, respectively.

DISCUSSION

We found previously that a forest bathing trip, but not a city visit significantly increased human NK activity, number of NK cells, and intracellular levels of perforin, GRN, and granzymes A/B in PBLs in male subjects (2-3), and that the increased NK activity lasted for more than 7 days after the trip (3). However, it was not clear whether a forest bathing trip can also increase NK activity in female subjects. The present study found that a forest bathing trip also enhances the immune response in the female subjects as measured by human NK activity and the percentage of NK cells, indicating that forest bathing does indeed enhance human NK activity. Moreover, we also confirmed that the increased NK activity and percentage of NK cells induced by a forest bathing trip lasted for more than 7 days, even 30 days, after the trip (3). This suggests that if people visit a forest once a month, they may be able to maintain increased NK activity. This may be important in health promotion and preventive medicine (3).

NK cells kill tumor or virus-infected cells by the release of perforin, granzymes (15, 18-21), and GRN (22-23) via the granule exocytosis pathway.

In order to explore the mechanism of enhancement of NK activity by forest bathing in female subjects, we investigated the effect of forest bathing on the intracellular levels of perforin, GRN, and GrA/B in PBLs. We found that the forest bathing trip significantly increased the proportion of PBLs expressing these effector molecules, confirming our previous reports (2-3). Moreover, we found that increased perforin, GRN, and GrA/B-expressing cells induced by a forest bathing trip lasted for more than 7 days after the trip, confirming our previous report (3). These cytolytic molecules contribute to NK and anti-tumor activity (23-24).

Adrenaline is released from the adrenal medulla, and the adrenaline level increases under circumstances of novelty, anticipation, unpredictability, and general emotional arousal, whereas noradrenaline is the predominant neurotransmitter released by the sympathetic system, and some of this enters the blood; the level

Table I. *Concentration of volatile substances (phytoncides) in the air of forest fields calculated as alpha-pinene (ng/m³).*

Measuring points	Field 1 Day 1 pm	Field 2 Day 2 am	Field 3 Day 2 pm
Kind of Trees	*Cryptomeria japonica D.Don*	*Cryptomeria japonica D.Don*	*Quercus mongolica var. grosseserrata, Fagus crenata Blume, Cercidiphyllum japonicum*
isoprene	3.8	8.0	12.8
tricyclene	1.6	3.0	2.1
α-pinene	103.7	35.7	13.2
camphene	14.1	2.7	1.3
β-pinene	4.6	26.7	5.8
δ-3-carene	1.6	0.0	0.0
p-cymene	1.6	0.0	0.0
limonene	1.6	0.0	0.0

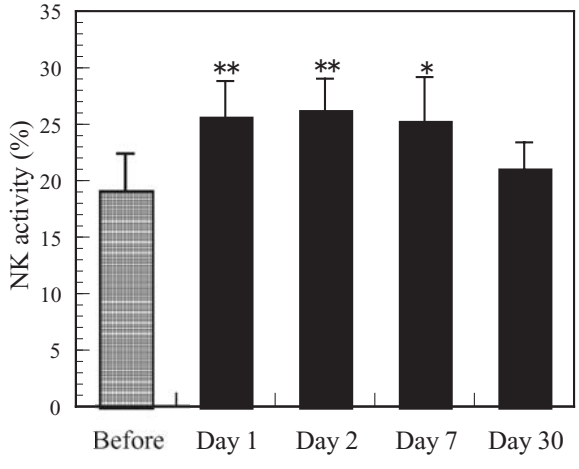

Fig. 1. *Effect of the forest bathing trip on NK activity. Data are presented as the mean+SE (n=13). Two-way ANOVA with no-repeated measures indicated that the forest bathing trip and the variability between individuals significantly affected NK activity (all p<0.01). *: p<0.05, **: p<0.01, significantly different from before the trip by paired t-test. The activity values for an E/T ratio of 20/1 are shown, and similar results were also obtained with E/T ratios of 40/1 and 10/1.*

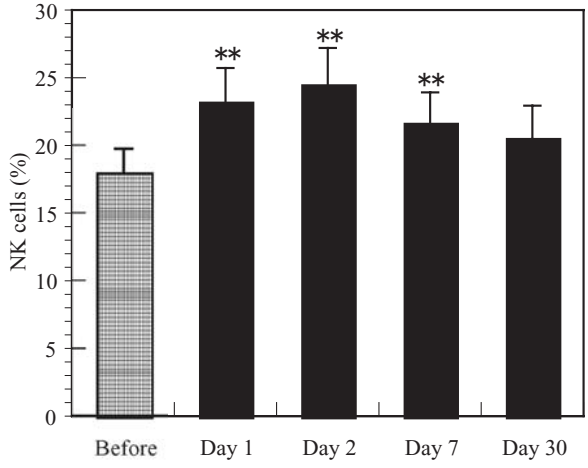

Fig. 2. *Effect of the forest bathing trip on the percentage of NK cells. Data are presented as the mean+SE (n=13). Two-way ANOVA with no-repeated measures indicated that the forest bathing trip and the variability between individuals significantly affected the percentage of NK cells (all p<0.01). *: p<0.05, **: p<0.01 significantly different from before the trip by paired t-test.*

of noradrenaline increases during increased physical activity (25). Measurement of free adrenaline and noradrenaline in urine provides a reliable measure of the circulating concentration of adrenaline and noradrenaline in the bloodstream and thus a measure of sympathoadrenal medulla activity (26). The concentrations of adrenaline and noradrenaline in urine have been used to evaluate work related stress in nurses (27), lorry drivers (28), long distance coach drivers (29), and psychosocial stress (30). We found

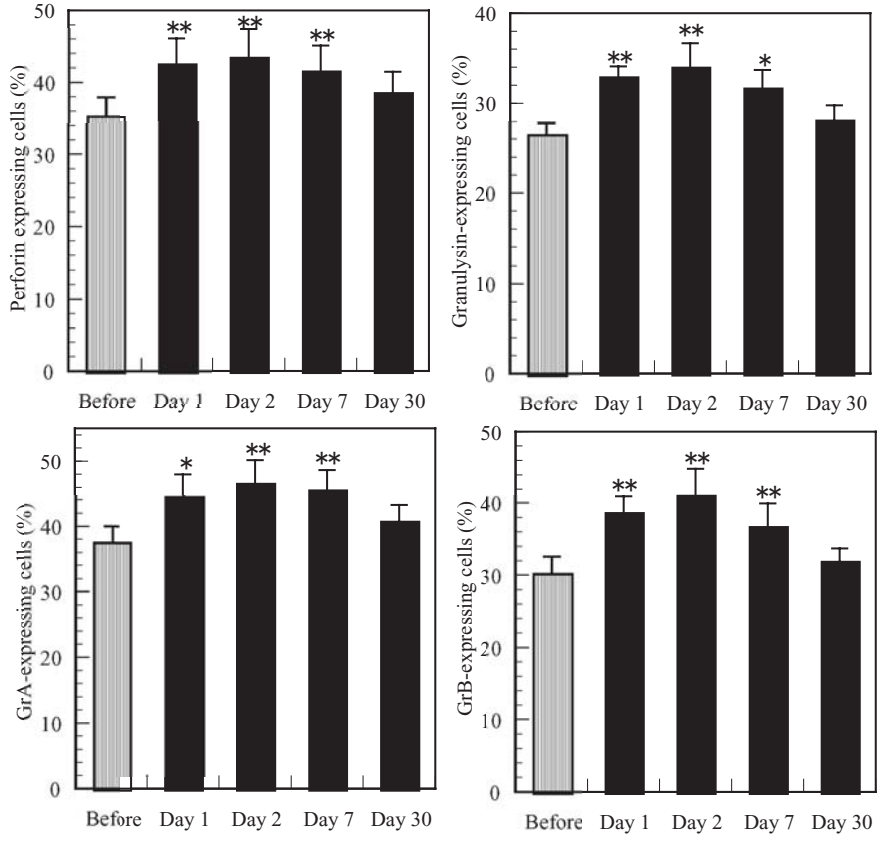

Fig. 3. *Effect of the forest bathing trip on GRN, perforin, and GrA/B-expressing cells in PBLs. Data are presented as the mean+SE (n=13). Two-way ANOVA with no-repeated measures indicated that the forest bathing trip and the variability between individuals significantly affected the GRN, perforin, GrA/B-expressing cells in PBLs (all p<0.01). *: p<0.05, **: p<0.01, significantly different from before the trip by paired t-test.*

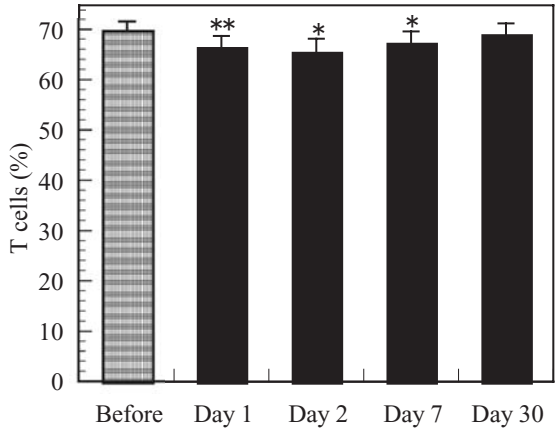

Fig. 4. *Effect of the forest bathing trip on the percentage of T cells. Data are presented as the mean+SE (n=13). Two-way ANOVA with no-repeated measures indicated that the forest bathing trip and the variability between individuals significantly affected the percentage of T cells (all p<0.05). *: p<0.05, **: p<0.01 significantly different from before the trip by paired t-test.*

that a forest bathing trip significantly decreased the concentrations of adrenaline and noradrenaline in urine, confirming our previous report (3), and suggesting that the subjects were under conditions of lower stress during the forest bathing trip. It has been reported that adrenaline inhibits human NK activity (31). Addition of noradrenaline to intrathecal morphine augments the postoperative suppression of natural killer cell activity (32), suggesting that noradrenaline also inhibits human NK activity. We found previously that physical and/or psychological stress decreased NK activity, NK receptor levels, and mRNA transcription of granzymes and perforin in mice (33). The increase in NK activity during a forest bathing trip may be related to an attenuated stress hormone response (adrenaline, noradrenaline) associated with the forest bathing trip, whereas increased sympathetic activity may

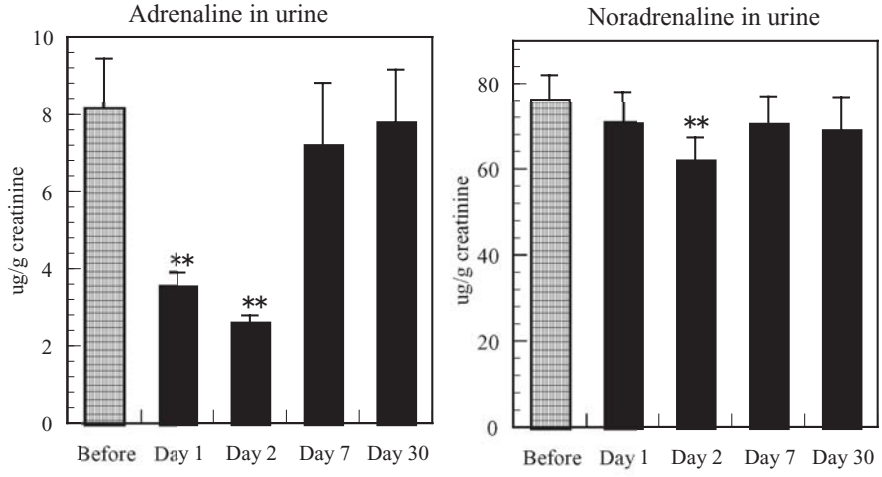

Fig. 5. *Effect of the forest bathing trip on adrenaline and noradrenaline concentrations in urine. Data are presented as the mean+SE (n=13). Two-way ANOVA with no-repeated measures indicated that the forest bathing trip significantly affected the adrenaline concentration in urine (p<0.01). *: p<0.05, **: p<0.01, significantly different from before the trip by paired t-test.*

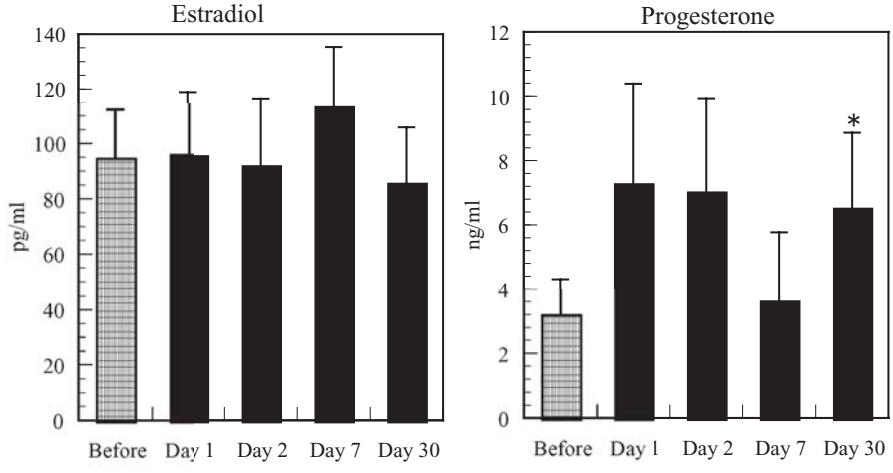

Fig. 6. *Changes in estradiol and progesterone concentrations in serum before, during, and after the trip. Data are presented as the mean+SE (n=13). Two-way ANOVA with no-repeated measures indicated that there was no difference in the estradiol and progesterone concentrations in serum between the days before, during, and after the trip. *: p<0.05, significantly different from before the trip by paired t-test.*

have an immunosuppressive effect through release of adrenaline (34). Previous studies have reported that a forest bathing trip reduces the concentration of cortisol in saliva, reduces prefrontal cerebral activity, reduces blood pressure, and stabilizes autonomic nervous activity in humans (35-37). The result of the POMS scores in the present study also suggests that the subjects were physiologically relaxed during the forest bathing trip.

We found that the forest bathing trip significantly decreased T cells in the female subjects. It has been reported that mental stress increased T cell in PBLs (38-40). We also have found that people with a poor lifestyle showed a higher percentage of T cells than people with a good lifestyle (5); therefore, we speculate that the proportion of T cells in PBLs may reflect stress status.

Many factors, including circadian variation (8-9), physical exercise (5, 41), alcohol consumption (5, 42), and menstruation (6) can affect human NK

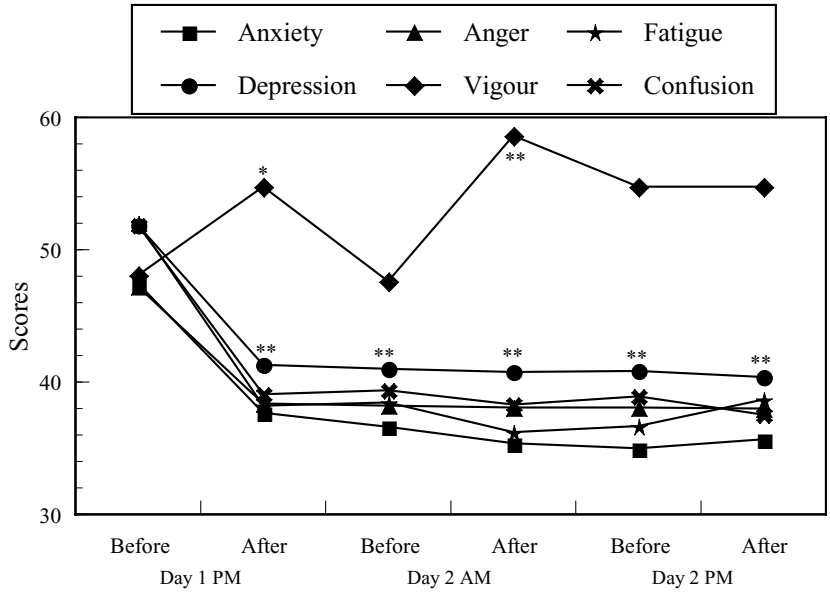

Fig. 7. *Effect of the forest bathing trip on POMS scores. Data are presented as the means (n=13). *: p<0.05, **: p<0.01, significantly different from before the trip on Day 1pm by paired t-test (anxiety, depression, anger, fatigue, and confusion).*

activity in female subjects. In order to control the effect of circadian rhythm on NK activity, we sampled blood at 8 am on all days. To control for the effect of physical exercise on NK activity, we limited the walking steps during the trip to the averaged normal workday distances as monitored by a pedometer. To control the effect of alcohol on NK activity, the subjects did not consume alcohol during the study period. The sleeping hours during the trips were a little longer than on average working days (data not shown). We found previously that slightly longer sleeping hours during the trip than those on average working days did not affect either NK activity, cell numbers, or the levels of perforin, GRN, or GrA/B-expressing cells in PBLs (2-3), and that there was no difference in the numbers of NK cells, nor the levels of perforin, GRN, or GrA/B-expressing cells in PBLs among the subjects who slept for 5, 6, or 7 hours (5). Kusaka et al. (43) also reported that sleeping hours did not affect NK activity, or NK cell numbers under physiological conditions.

It has been reported that NK activity was significantly higher in the follicular than in the luteal phase of the menstrual cycle, and that postmenopausal women showed NK activity similar to women in the follicular phase but significantly higher than women in the luteal phase of the menstrual cycle (6). On the other hand, Yovel et al. (44) reported that the menstrual cycle had no significant effect on activity levels of NK cells. Roszkowski et al. (10) found that patients with low (<50 pg/ml) and high (>200 pg/ml) estradiol levels showed an increase and a decrease of NK cell activity, respectively. Progesterone at 100-400 nM (31.45-125.8 ng/ml) inhibits NK activity in healthy pregnant women, whereas 100 times higher concentrations are required for reducing NK activity in non-pregnant women (11). On the other hand, estradiol at 10^{-6} M (272.39 pg/ml) or progesterone at 10^{-6} M (314.47 pg/ml) did not affect NK activity with 20 h in vitro incubation (12); no significant correlation between the increase in NK activity and the decrease in estradiol concentration was found (13). The above mentioned studies suggest that the menstrual cycle and the levels of estradiol and progesterone in serum may affect human NK activity. To control for the influence of menstrual cycle on NK activity, we took a questionnaire to obtain information on the menstrual cycle of the subjects. The ratios of subjects who were in the follicular phase during the experiment were 5/13, 6/13, 6/13, 7/13, and 6/13 on the day before the trip, days 1 and 2 during the trip, and days 7 and 30 after

the trip, respectively, indicating that there was no significant difference in the menstrual cycle of the subjects between the different days. This suggests that the menstrual cycle had a similar influence on NK activity on the different days. In addition, we also measured the concentrations of estradiol and progesterone in serum of the subjects to confirm the influence of estradiol and progesterone on NK activity. In the present study, there was no significant difference in the concentration of estradiol in serum between days before, during, and after the forest bathing trip, indicating that estradiol had a similar effect on NK activity between different days in the subjects in this case. Although there was no significant difference in the concentration of progesterone in serum between days 1 or 2 and before the trip, the levels of progesterone on days 1 and 2 were higher than that before, suggesting that progesterone may show an inhibitory effect on NK activity on days 1 and 2. However, although there was a possible inhibitory effect of progesterone, the NK activity on days 1 and 2 was still significantly higher than that before, indicating that the effect of the forest bathing trip on NK activity has exceeded the effect of progesterone. The level of progesterone on day 30 was significantly higher than that before the trip, suggesting that progesterone may show an inhibitory effect on NK activity on day 30. In fact, we found previously that increased human NK activity lasted for more than 30 days in male subjects (3); however, in the present study, although the NK activity on day 30 was higher than that before the trip, the difference was not significant, suggesting that this may be due to the higher level of progesterone in serum in the subjects on day 30.

As detailed in Table I, we detected several phytoncides, such as alpha-pinene, beta-pinene, tricyclene, camphene, and limonene in the forest fields during the trip, but not in the urban area of Tokyo. We found previously that such phytoncides significantly enhanced human NK activity and increased the expression of intracellular cytolytic molecules, perforin, GrA, and GRN *in vitro* (1). Komori et al. (4) also reported that citrus fragrance found in forest affects the human endocrine and immune systems as analyzed by the measurement of urinary cortisol and dopamine levels, NK activity, and CD4/8 ratios. These findings suggest that phytoncides may contribute to the enhanced NK activity during the forest bathing trip (3).

In summary, a forest bathing trip can increase NK activity, the number of NK cells, and the expression of intracellular perforin, GrA/B, and GRN in female subjects. Forest bathing may contribute to decreased stress and improved immunity, and phytoncides from trees may contribute to this effect.

ACKNOWLEDGEMENTS

This work was supported in part by research projects from Japanese Society of Forest Therapy and Shinano Town, Nagano prefecture, Japan (2007).

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