FOREST BATHING ENHANCES HUMAN NATURAL KILLER ACTIVITY AND EXPRESSION OF ANTI-CANCER PROTEINS

Q. LI, K. MORIMOTO[1], A. NAKADAI, H. INAGAKI, M. KATSUMATA, T. SHIMIZU, Y. HIRATA, K. HIRATA, H. SUZUKI, Y. MIYAZAKI[2], T. KAGAWA[2], Y. KOYAMA[3], T. OHIRA[2], N. TAKAYAMA[2], A.M. KRENSKY[4] and T. KAWADA

Department of Hygiene and Public Health, Nippon Medical School, Tokyo;
[1]Department of Social and Environmental Medicine, Osaka University Graduate School of Medicine, Osaka;
[2]Forestry and Forest Products Research Institute, Tsukuba, Ibaraki; [3]Nagano Prefecture Forest Research Center, Nagano, Shiojiri, Japan; [4]Stanford University School of Medicine, Palo Alto, CA, USA

In order to explore the effect of forest bathing on human immune function, we investigated natural killer (NK) activity; the number of NK cells, and perforin, granzymes and granulysin-expression in peripheral blood lymphocytes (PBL) during a visit to forest fields. Twelve healthy male subjects, age 37-55 years, were selected with informed consent from three large companies in Tokyo, Japan. The subjects experienced a three-day/two-night trip in three different forest fields. On the first day, subjects walked for two hours in the afternoon in a forest field; and on the second day, they walked for two hours in the morning and afternoon, respectively, in two different forest fields. Blood was sampled on the second and third days, and NK activity; proportions of NK, T cells, granulysin, perforin, and granzymes A/B-expressing cells in PBL were measured. Similar measurements were made before the trip on a normal working day as the control. Almost all of the subjects (11/12) showed higher NK activity after the trip (about 50% increased) compared with before. There are significant differences both before and after the trip and between days 1 and 2 in NK activity. The forest bathing trip also significantly increased the numbers of NK, perforin, granulysin, and granzymes A/B-expressing cells. Taken together, these findings indicate that a forest bathing trip can increase NK activity, and that this effect at least partially mediated by increasing the number of NK cells and by the induction of intracellular anti-cancer proteins.

A forest bathing trip, called "Shinrinyoku" in Japanese, involves a visit to a forest field for the purpose of relaxation and recreation. It has been reported that forest bathing trips can decrease blood glucose and blood pressure (1), reduce the concentration of cortisol in saliva, reduce prefrontal cerebral activity and stabilize autonomic nervous activity in humans (2). In addition, citrus fragrance found in the forest affects the human endocrine and immune systems as analyzed by measurement of urinary cortisol and dopamine levels, natural killer (NK) activity and CD4/8 ratios (3). We previously reported that phytoncides enhanced human NK activity and intracellular levels of perforin, granulysin and granzyme A in NK cells *in vitro* (4). Although these findings strongly suggest that forest bathing trip may have beneficial effects on human immune function, there have been no reports to date investigating the effect of forest bathing on human NK activity.

NK and cytotoxic T lymphocyte (CTL) cells induce tumor cell death by two main mechanisms (5). One mechanism involves granule exocytosis, with the direct release of cytolytic granules containing perforin, granzymes (6-8), and granulysin (9-10) that kill target cells via apoptosis. A second mechanism involves receptor-ligand interactions between Fas and Fas ligand (FasL) (5, 11-12).

To test the effect of forest bathing on human immune function, we investigated NK activity; the proportions of NK and T cells, and perforin, granzymes and granulysin-expression in human peripheral blood lymphocytes during a visit to forest fields.

MATERIALS AND METHODS

Subjects. Twelve healthy male subjects, aged 37-55 years (43.1 ± 6.1), were selected from three large companies in Tokyo, Japan in the present study. The information gathered from a self-administered questionnaire including age, and lifestyle habits that asked about cigarette smoking, alcohol drinking habits, eating breakfast, sleeping hours, working hours, physical exercise, nutritional balance and mental stress, which have been reported previously (13-14). 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

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

Mailing address: Qing Li, M.D., Ph.D.
Department of Hygiene and Public Health,
Nippon Medical School,
1-1-5 Sendagi, Bunkyo-ku,
Tokyo 113-8602, Japan
Tel: ++81-3-3822-2131 Fax: ++81-3-5685-3065
e-mail: qing-li@nms.ac.jp

0394-6320 (2007)

analysis, or were taking any medications at the time of the study. The study was approved by the Ethics Committee of Nippon Medical School (approval No. 16-1).

Forest bathing trip. The subjects experienced a three-day/two-night trip at three different forest fields in early September, 2005. On the first day, subjects walked for 2 hours in the afternoon in a forest field, and then stayed at a nearby hotel within the forest. On the second day, subjects walked for 2 hours in the morning and afternoon, respectively, in two different forest fields. Each course was 2.5 km, closely resembling normal physical activity for the subjects on normal working days. Daily physical activity of the subjects was monitored with a pedometer and 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. The validation study was previously reported (15). Blood was sampled on the second and third days and three days prior to the trip as a control. Since it has been reported that human NK cell activity shows circadian rhythms (16), all samples were obtained at 8:00 am. All blood samples were placed in an ice/water box at 4°C and assays performed within four hours of the blood draw. NK activity; proportions of NK, T cells, granulysin, perforin, and granzymes A/B-expressing cells in peripheral blood lymphocytes (PBL) were measured.

Reagents. RPMI 1640 medium was purchased from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from JRH Biosciences (Lenexa, KS). Sodium ^{51}Cr-chromate was obtained from PerkinElmer (Boston, MA). Fluorescein isothiocynate (FITC)-mouse anti-human perforin, granzyme A (GrA), granzyme B (GrB) and FITC/phycoerythrin (PE)-CD16, PerCP-Cy5.5-CD3, FITC/PE-negative isotypic control antibodies, and Cytofix/cytoperm solution were purchased from BD Pharmingen (San Diego, CA). Rabbit anti-human granulysin (GRN) polyclonal antibody was described previously (9). PE-goat-anti rabbit IgG were purchased from Vector Laboratories Inc. (Burlingame, CA).

NK activity. Human PBL were separated from peripheral blood with BD Vacutainer CPT (Becton Dickinson, Franklin Lakes, NJ), and then adjusted to $4x10^6$ cells/ml for the assay of NK activity. Human NK activity was assayed according to the traditional method (8).

Cell staining and flow cytometric analysis. NK and T cells in PBL were stained with PE/FITC-CD16 and PerCP-Cy5.5-CD3 for 30 min in the dark. Then, the cells were fixed/permeablized with Cytofix/cytoperm solution for 20 min at 4°C, and then the intracellular perforin and GrA/B were stained with FITC- anti-human perforin and GrA/B, respectively, for 30 min at 4°C. Intracellular GRN was stained with rabbit anti-human GRN polyclonal Ab after fixation/permeablization with Cytofix/cytoperm solution, and then stained with PE-goat anti-rabbit IgG for 30 minutes at 4°C in the dark. Flow cytometric analysis was performed with a flow cytometer as described previously (17).

White blood cell (WBC) count. WBC and the percentages of granulocyte, lymphocyte and monocyte were determined by an automatic cell counter.

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 (18).

Measurements of phytoncide, and environmental temperature/ humidity in the forest fields during the investigation. The volatile organic compounds in forest air were trapped with glass cartridges, which were filled with adsorbent. The sampler was set at 1.2m above the ground and total amounts of 138.6-162.0 L of forest air were pumped through during 23.1-24.7 hrs. The loaded cartridges were stored at 4°C and analyzed within 7 days. The volatile organic compounds such as alpha-pinene, beta-pinene and isoprene were measured with an ATD 400 automatic thermodesorption (Perkin Elmer, Boston, MA) device coupled with GC-MS (Agilent Technologies, CA). The components were identified by GC-MS equipped with a selected ion monitoring (SIM) functions as described previously (19).

Temperature and humidity in the forest fields were measured with an Amenity-Meter (AM-101, Kyoto Electronics Manufacturer CO., LTD. Kyoto, Japan) as described previously (20).

Statistical analysis. Multiple comparisons were made with the paired t-test if the analysis of variance was significant. The analysis was performed with the Microsoft Excel software package for Windows. The significance level for p values was set at < 0.05.

RESULTS

Effect of forest bathing trip on WBC. As shown in Table I, the forest bathing trip significantly increased the percentages of lymphocytes and monocytes, and significantly decreased the percentages of granulocytes in the peripheral blood of the subjects. The forest bathing trip did not affect WBC counts.

Effect of forest bathing trip on NK activity and the percentage/number of NK ($CD16^+$) cells. Eleven of twelve subjects displayed increased NK activity during and after the trip as compared to three days before. Significant differences were observed both before and after the trip and between days 1 and 2 in NK activity (Fig. 1A). Forest bathing also significantly increased NK cells in all subjects, with significant differences before and after the trip and between days 1 and 2 in both the percentage (Fig. 1B) and total number (Fig. 1C) of NK cells.

Effect of forest bathing trip on the percentage/number of cells expressing cytolytic molecules. The forest bathing trip also significantly increased the percentages and total number of GRN, perforin, and GrA/B-expressing cells in PBL (Fig. 2).

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

The forest bathing trip significantly decreased the percentage of T cells, but not the total number of T cells (data not shown).

Effect of forest bathing trip on the score of POMS test. In addition, the forest bathing trip significantly increased the score for vigour and decreased the scores for anxiety, depression and anger. There was no significant change in the scores for fatigue or confusion in POMS (Fig. 3).

There were no significant differences in walking steps before and during the trip (before: 9614, day 1: 10470, Day 2: 9328 steps). The hours of sleep were, however, increased during the trip compared with the control (day 1: 7.56 ± 1.27, day 2: 7.21 ± 1.44, before: 6.38 ± 0.88 hours).

Lastly, phytoncides, such as alpha-pinene (17.4-812.6 ng/m^3), beta-pinene (2.3-41.6 ng/m^3) and isoprene (10.7-10850.8 ng/m^3) were detected in the forest fields during the investigation, and not detected in the urban area of Tokyo. Weather during the forest bathing trip was excellent with average temperatures and humidity in the forest fields during the walking of 23.4 ± 0.6°C, 87.7 ± 3.4% on day 1 in the afternoon; 21.9 ± 1.0°C, 84.5 ± 4.8% on day 2 in the morning; and 25.8 ± 1.0°C, 77.4 ± 6.1% on day 2 in the afternoon, The average temperature and humidity in urban area of Tokyo on the control day was 26.7°C, 58%, respectively.

DISCUSSION

The present study demonstrates that a forest bathing trip can enhance the immune response as measured by human NK activity, and the percentage and absolute numbers of NK cells. This is the first report to investigate the direct effect of forest bathing on human NK activity and numbers.

NK cells kill tumor cells by release of perforin, granzymes (Gr) (6-8), and granulysin (9-10) via the granule exocytosis pathway. Cytotoxicity mediated by NK cells is greatly impaired in perforin-deficient mice (11-12). GrA plays a critical role in triggering apoptosis in target cells either directly or *via* the activation of cellular caspases, and also cleaves IL-1β, the nucleosome assembly protein called putative HLA-associated protein II, TAF-Iβ, and lamins (6-7, 21). GrB directly cleaves the downstream caspase substrates, nuclear matrix antigen, catalytic subunit of DNA-associated DNase inhibitor and lamins (6, 21). GRN, a lytic molecule expressed by human CTL and NK cells, is active against tumor cells and a variety of microbes. GRN can enter target cells in the absence of perforin and induce apoptosis, although GRN and perforin together are required to kill intracellular microbes like *Mycobacteria tuberculosis* (9-10).

In order to explore the mechanism of enhancement of NK activity by forest bathing, we investigated the effect of forest bathing on the intracellular levels of perforin, GRN, and GrA/B in PBL. We found that the forest bathing trip significantly increased both the proportion and number of perforin, GRN, GrA/B-expressing cells in PBL. These cytolytic molecules contribute to NK and anti-tumor activity.

It has been reported that dominance by the parasympathetic nervous system causes an increase in circulating lymphocytes and decrease in granulocytes in peripheral blood (22). We found that forest bathing significantly increased the proportions of lymphocytes and monocytes and decreased the proportions of granulocytes in WBC, suggesting that the parasympathetic nervous system of subjects was dominant, associated with relaxation and decreased stress. Previous studies have reported that forest bathing reduces the concentration

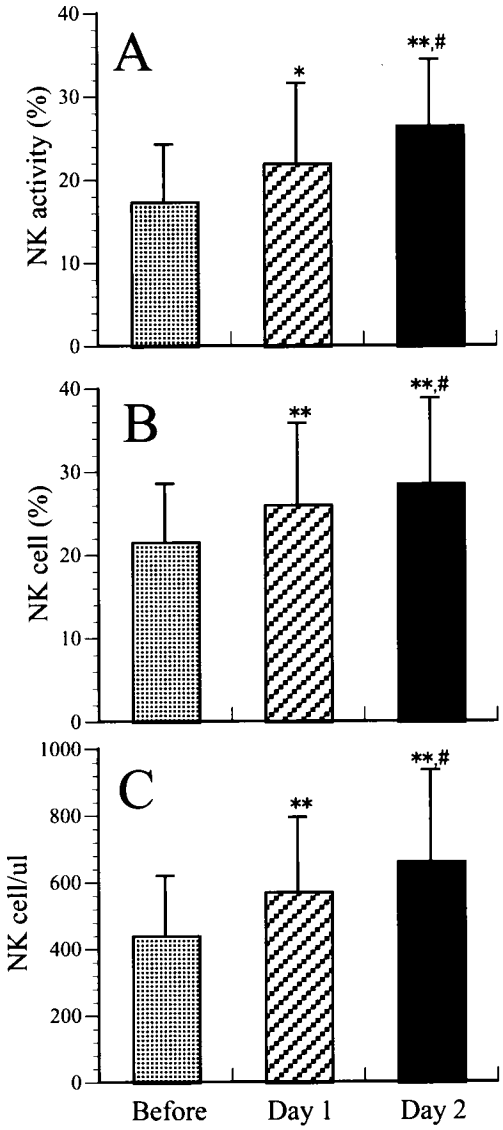

Fig. 1. *Effect of the forest bathing trip on NK activity (A), the percentages (B) and total number (C) of NK cells. Data are presented as the mean+SD (n=12). ANOVA indicated that the forest bathing trip significantly affected the NK activity, the percentages and total number of NK cells (all p<0.01). *: p<0.05, **: p<0.01, significantly different from before the trip, #: p<0.05 significantly different from Day 1 by the paired t-test. The activity values for an E/T ratio of 20/1 are shown, and the similar results were also obtained with E/T ratios of 40/1 and 10/1.*

of cortisol in saliva, reduces prefrontal cerebral activity, reduce blood pressure and stabilize autonomic nervous activity in humans (1-2). The result of the POMS score in the present study also suggests that the subjects were physiologically relaxed during the forest bathing trip.

Although the forest bathing trip significantly decreased the percentage of T cells, the absolute number of T cells was unchanged before and after the forest bathing. Thus the decrease in T cell percentage was due to an increase in

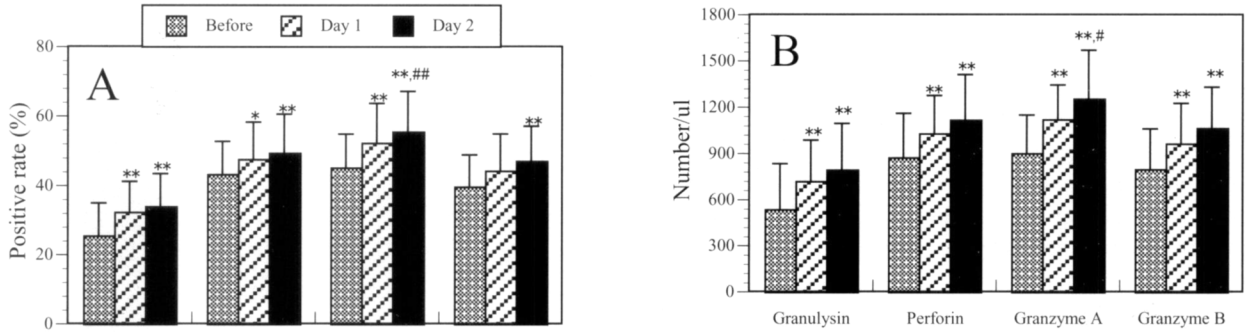

Fig. 2. *Effect of the forest bathing trip on the proportion (A) and number (B) of GRN, perforin, GrA/B-expressing cells in PBL. Data are presented as the mean +SD (n=12). ANOVA indicated that the forest bathing trip significantly affected the proportion and number of GRN, perforin, GrA/B-expressing cells in PBL (all p<0.01). *: p<0.05, **: p<0.01, significantly different from before the trip, #: p<0.05, ##: p<0.01 significantly different from Day 1 by the paired t-test.*

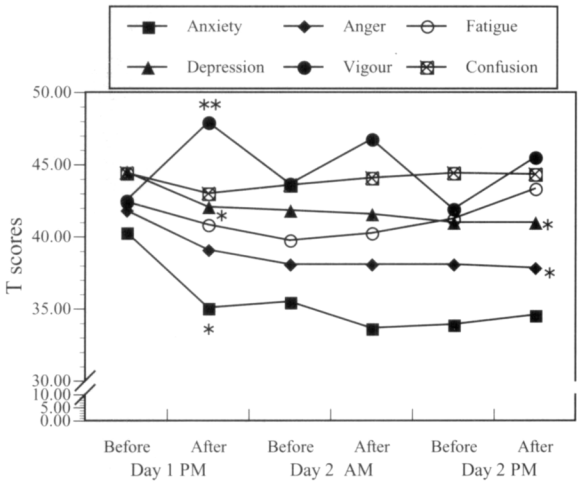

Fig. 3. *Effect of the forest bathing trip on POMS scores. *: p<0.05, **: p<0.01, significantly different from before the trip on Day 1pm by the paired t-test.*

Table I. *Effect of the forest bathing trip on the populations of WBC (Mean±SD).*

	Before	Day 1	Day 2
WBC (/ul)	6533±1559	6325±1042	6467±1157
Lymphocytes (%)	31.45±5.46	34.93±5.41*	35.47±3.88**
Monocytes (%)	4.27±0.85	4.33±0.83	4.91±0.94**,#
Granulocytes (%)	64.28±5.71	60.75±5.29*	59.63±3.88**

**: p<0.05, **: p<0.01 significantly different from before the trip, #: p<0.01 significantly different from Day 1 by paired t-test*

NK cell numbers.

Many factors, including circadian variation (16), physical exercise (23) and alcohol consumption (24) can affect human NK activity. In order to control the effect of circadian rhythms 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 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. The sleeping hours during the trip were a little longer (day 1: 7.56 ± 1.27, day 2: 7.21 ± 1.44 hours) than the average working day (before: 6.38 ± 0.88 hours). There are several reports addressing the effect of sleeping hours on NK cell activity. Many reports suggest that sleep deprivation increases human NK activity (25), while others suggest that sleep deprivation decreased human NK activity (26); still other studies by Kusaka et al. (13) and Inoue et al., (14) reported that sleeping hours did not affect NK or LAK activity, or NK cell numbers under physiologic conditions. In fact, we also found that there was no difference in the number of NK cells, or perforin, GRN, GrA/B-expressing cells in PBL among the subjects who slept 5, 6 or 7 hours, respectively (27). Taken together, although the sleeping hours during the trip were a little longer than that on the average working day, this difference did not affect either NK activity or numbers in the present study.

We detected several phytoncides such as alpha-pinene, beta-pinene and isoprene in the forest fields during the trip. We previously found that phytoncides, such as alpha-pinene, d-limonene significantly enhance human NK activity and increase expression of intracellular cytolytic molecules, perforin, GrA and GRN *in vitro* (4), suggesting that phytoncide may partially contribute to the enhanced NK activity during the forest bathing trip.

Taken together, these findings indicate that forest bathing can increase human NK activity, and that this effect at least partially mediated by the induction of intracellular perforin, GrA/B and GRN and increased number of NK cells.

ACKNOWLEDGEMENTS

This work was supported partly by a research project

for utilizing advanced technologies in agriculture, forestry and fisheries of Japan (2005) and by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (No.16107007).

REFERENCES

1. **Ohtsuka Y., N. Yabunaka and S. Takayama.** 1998. Shinrin-yoku (forest-air bathing and walking) effectively decreases blood glucose levels in diabetic patients. *Int. J. Biometeorol. 41:125.*
2. **Park B.J., Y. Tsunetsugu, T. Kasetani, T. Ohira, N. Matsui, N. Takayama, H. Murata, M. Yamaguchi, A. Yasukouchi, H. Hirano, T. Kagawa and Y. Miyazaki.** 2005. Physiological effects of bathing in the forest atmosphere (I)—Using salivary cortisol and cerebral activity (TRS) as an indicator. *J. Physiol. Anthropol. Appl. Human Sci. 24:188.*
3. **Komori T., R. Fujiwara, M. Tanida, J. Nomura and M.M. Yokoyama.** 1995. Effects of citrus fragrance on immune function and depressive states. *Neuroimmunomodulation 2:174.*
4. **Li Q., A. Nakadai, H. Matsushima, Y. Miyazaki, A.M. Krensky, T. Kawada and K. Morimoto.** 2006. Phytoncides (wood essential oils) induce human natural killer cell activity. *Immunopharmacol. Immunotoxicol. 28: 319.*
5. **Kagi D., F. Vignaux, B. Ledermann, K. Burki, V. Depraetere, S. Nagata, H. Hengartner and P. Golstein.** 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science 265:528.*
6. **Smyth M.J., J. M. Kelly, V.R. Sutton, J.E. Davis, K.A. Browne, T.J. Sayers and J. A. Trapani.** 2001. Unlocking the secrets of cytotoxic granule proteins. *J. Leukoc. Biol. 70:18.*
7. **Beresford P.J., C.M. Kam, J.C. Powers and J. Lieberman.** 1997. Recombinant human granzyme A binds to two putative HLA-associated proteins and cleaves one of them. *Proc. Natl. Acad. Sci. USA 94:9285.*
8. **Li Q., N. Nagahara, H. Takahashi, K. Takeda, K. Okumura and M. Minami.** 2002. Organophosphorus pesticides markedly inhibit the activities of natural killer, cytotoxic T lymphocyte and lymphokine-activated killer: a proposed inhibiting mechanism via granzyme inhibition. *Toxicology 172:181.*
9. **Hanson D.A., A.A. Kaspar, F.R. Poulain and A.M. Krensky.** 1999. Biosynthesis of granulysin, a novel cytolytic molecule. *Mol. Immunol. 36:413.*
10. **Stenger S., D.A. Hanson, R. Teitelbaum, P. Dewan, K.R. Niazi, C.J. Froelich, T. Ganz, S. Thoma-Uszynski, A. Melian, C. Bogdan, S.A. Porcelli, B.R. Bloom, A.M. Krensky and R.L. Modlin.** 1998. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science 282:121.*
11. **Kagi D., B. Ledermann, K. Burki, P. Seiler, B. Odermatt, K.J. Olsen, E.R. Podack, R.M. Zinkernagel and H. Hengartner.** 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature 369:31.*
12. **Li Q., A. Nakadai, K. Takeda and T. Kawada.** 2004. Dimethyl 2,2-dichlorovinyl phosphate (DDVP) markedly inhibits activities of natural killer cells, cytotoxic T lymphocytes and lymphokine-activated killer cells *via* the Fas-ligand/Fas pathway in perforin-knockout (PKO) mice. *Toxicology 204:41.*
13. **Kusaka Y., H. Kondou and K. Morimoto.** 1992. Healthy lifestyles are associated with higher natural killer cell activity. *Prev. Med. 21:602.*
14. **Inoue C., T. Takeshita, H. Kondo and K. Morimoto.** 1996. Healthy lifestyles are associated with higher lymphokine-activated killer cell activity. *Prev. Med. 25: 717.*
15. **Kawada T., P. Xin, M. Kuroiwa, Y. Sasazwa, S. Suzuki and Y. Tamura.** 2001. Habituation of sleep to road traffic noise as determined by polysomnography and accelerometer. *J. Sound Vib. 242:169.*
16. **Angeli A.** 1992. Circadian rhythms of human NK cell activity. *Chronobiologia 19:195.*
17. **Li Q., A. Nakadai, M. Ishizaki, K. Morimoto, A. Ueda, A.M. Krensky and T. Kawada.** 2005. Dimethyl 2,2-dichlorovinyl phosphate (DDVP) markedly decreases the expression of perforin, granzyme A and granulysin in human NK-92CI cell line. *Toxicology 213:107.*
18. **Yokoyama K., S. Araki, N. Kawakami and T. Takeshita.** 1990. Production of the Japanese edition of the Profile of Mood States (POMS): assessment of reliability and validity. *Nippon Koshu Eisei Zasshi 37:913.*
19. **Yokouchi Y., T. Fujii, Y. Ambe and K. Fuwa.** 1981. Determination of monoterpene hydrocarbons in the atmosphere. *J. Chromatogr. 209:293.*
20. **Takayama N., T. Kagawa, Y. Tsunetsugu, B. Park, Y. Oishi and T. Kasetani.** 2004. Measurement and comparison of warm temperature environment of summer forest inside and outside using PMV (Predicted Mean Vote). *Thesis Collection on Kanto Branch of the Japanese Forest Society 56:21.*
21. **Zhang D., P.J. Beresford, A.H. Greenberg and J. Lieberman.** 2001. Granzymes A and B directly cleave lamins and disrupt the nuclear lamina during granule-mediated cytolysis. *Proc. Natl. Acad. Sci. USA 98:5746.*

22. **Mori H., K. Nishijo, H. Kawamura and T. Abo.** 2002. Unique immunomodulation by electro-acupuncture in humans possibly via stimulation of the autonomic nervous system. *Neurosci. Lett. 320:21.*

23. **Nieman D.C.** 2000. Special feature for the Olympics: effects of exercise on the immune system: exercise effects on systemic immunity. *Immunol. Cell Biol. 78:496.*

24. **Ochshorn-Adelson M., G. Bodner, P. Toraker, H. Albeck, A. Ho and M.J. Kreek.** 1994. Effects of ethanol on human natural killer cell activity: *in vitro* and acute, low-dose *in vivo* studies. *Alcohol Clin. Exp. Res. 18:1361.*

25. **Matsumoto, Y., K. Mishima, K. Satoh, T. Tozawa, Y. Mishima, T. Shimizu, and Y. Hishikawa.** 2001. Total sleep deprivation induces an acute and transient increase in NK cell activity in healthy young volunteers. *Sleep 24: 804.*

26. **Moldofsky H., F.A. Lue, J.R. Davidson and R. Gorczynski.** 1989. Effects of sleep deprivation on human immune functions. *FASEB J. 3:1972.*

27. **Li Q., K. Morimoto, A. Nakadai, T. Qu, H. Matsushima, M. Katsumata, T. Shimizu, H. Inagaki, Y. Hirata, K. Hirata, T. Kawada, Y. Lu, K. Nakayama, A.M. Krensky.** 2007. Healthy lifestyles are associated with higher levels of perforin, granulysin and granzymes A/B-expressing cells in peripheral blood lymphocytes. *Prev. Med. 44:117.*