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ORIGINAL ARTICLE

Tea tree oil inhalations modify immunity in mice

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Summary
Tea tree oil (TTO) is well known as an anti-microbial and immunomodulatory agent and the latter property was examined in this study. Male, $C_{57}BI_{10}$ x CBA/H (F1), mice were exposed to TTO vapour three times a day, for one week. During this period, half of the mice also received naltrexone (endogenous opioid receptor antagonist) in their drinking water. A day before the end of the TTO inhalation treatment a number of the mice were intra-peritoneally injected with Zymosan or PBS. Spleens and peritoneal exudates were collected 24 h after the injections. Cultured splenocytes were used in *in vitro* proliferation assays with PHA, and LPS mitogens and peritoneal leukocytes (PTLs) were used for cytofluorimetric ROS level measurement.

The results obtained confirmed the anti-inflammatory properties of TTO, expressed as an inhibition of the increase in the PTL number stimulated by Zymosan. This effect was reversed by naltrexone, suggesting that TTO acts via the endogenous opioid system. TTO also stopped the proliferation of splenocytes in response to mitogens and the activity of PTLs was equivalent to that seen in the control (without inflammation) groups.

Keywords: tea tree oil – peritonitis – splenocytes – immunity – mice

INTRODUCTION

Tea tree oil (TTO) is an essential oil distilled from an Australian native plant: the tea tree (*Melaleuca alternifolia*). This oil contains over a hundred different compounds, mainly monoterpenes and their derivatives. Well known as an antimicrobial agent, TTO is able to kill a wide range of bacteria, fungi and viruses, due to the presence of terpinen-4-ol, γ-terpinen and 1,8 – cineole, which are the main active components (Shnaubelt 1989, Carson et al. 1995, Schnitzler 2001, Hammer et al. 2002).

There is also evidence that TTO exerts immunomodulatory effects. The main components of TTO exhibit an anti-inflammatory activity *in vitro*, suppressing the production of pro-inflammatory cytokines by lipopolysaccharide (LPS)-activated human monocytes (Finlay-Jones et al. 2002a). The water-soluble fraction of this oil suppressed LPS-stimulated superoxide production by monocytes, but not by neutrophils (Finlay-Jones et al. 2002b). An anti-inflammatory effect of TTO has also been observed in *in vivo* studies. When topically applied, TTO reduced the oedema

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associated with a contact hypersensivity response to a chemical hapten (Brand et al. 2002a) and with the intradermal injection of histamine (Brand et al. 2002b), suggesting an inhibitory effect on proinflammatory cytokine production by lymphocytes.

On the other hand, data indicating an immuno-stimulatory effect of TTO inhalation are also available. Mice submitted to multiple sessions of TTO inhalation exhibited an increase in the level of circulating blood immunoglobulins and the blood granulocyte number plus stimulation of the local Graft-versus-Host reaction of spleen cells (Skopinska-Rozewska et al. 1997).

Neutrophils and macrophages are cells which play an important role at the site of inflammation, where they remove pathogenic microorganisms by the process of phagocytosis. Their anti-microbial activity results in high levels of ROS (Reactive Oxygen Species) being produced during inflammation, which may, however also be harmful to the host. Since peritoneal leukocytes are easy to collect and amenable to study (Ajuebor et al. 1998), experimental peritonitis is a good model for the investigation of inflammation, inducing the modulatory effects of various factors on this process (Chadzinska et al. 1999, Majewski et al. 2002).

The aim of this study was to examine the influence of multiple sessions of TTO inhalation on the number of peritoneal leukocytes and their ROS level in mice with experimental peritonitis as well as on *in vitro* proliferation of splenocytes stimulated with T- and B-cell mitogens. Additionally, the role of endogeonous opioids in the effect of TTO inhalations on the inflammatory reaction in mice was assessed.

MATERIALS AND METHODS

Animals

Experiments were performed on 6-8-week-old $C_{57}BI_{10}$ x CBA/H (F1) male mice. Before starting the experiment, animals were randomly divided into one experimental and two control groups and placed in a breeding room in separate cages with 6 – 9 individuals per cage. The mice were maintained under standard conditions: 22 – 24 ˚C, LD 12/12 with free access to standard food and water. Animals were treated according to Polish regulations concerning experiments on animals, and the procedure was approved by the local Ethical Commission.

Experimental design

TTO inhalation treatment

Treatment with TTO started after one week of acclimation to the new room and cages. Inhalation sessions were performed three times a day for 15 min each, for 7 days (adapted from Skopinska-Rozewska et al. 1997). Mice were transported to the laboratory three times a day for each inhalation session, in cages destined for transportation and placed into cages destined for inhalation to eliminate the distribution of TTO to the breeding room and to avoid an adaptation of receptors and neurons to the smell of TTO (Hildebrand and Sheferd 1997). Pieces of cotton wool moistened with water and five drops of TTO were placed in the closed plastic container with small holes which was put onto the cover of the cage. The mice had no access to the treated cotton wool. For the period of inhalation, the cages were covered with material (towel) to minimize draughts through the cage and to concentrate the vapours of TTO.

Experimental groups

In addition to the TTO inhalation treatment group (TTO) two control groups were created. One of them received no inhalation treatment (Control group), the other received sham inhalation treatment (Placebo group) and were submitted to the same procedure as mice from the TTO group, except that the cotton wool was moistened only with water. Mice from all of these groups (TTO, Control and Placebo) were injected with PBS, Zymosan or left untreated.

Induction of peritonitis and peritoneal leukocyte (PTL) isolation

Peritonitis was elicited 24 h before the end of the inhalation sessions. Mice were injected i.p. with 0,5 ml of Zymosan A solution (2mg/ml in phosphate-buffered saline – PBS) or with the same volume of PBS (Kolaczkowska et al. 2000). In each experimental and control group 3 –.4 mice were left untreated. After sacrifice by cervical dislocation, the peritoneal cavity was flushed out with 10 ml of heparinized PBS and the retrieved PTLs were counted and used for the measurement of ROS level.

Naltrexone treatment

Half the animals from each group were additionally submitted to treatment with naltrexone (opioid receptor antagonist) which was added to their drinking water at a concentration of 0.5 mg/ml (Moynihan et al. 2000). The average uptake of naltrexone administered in this way, was 1.1 mg per mouse / 24 h and did not differ between experimental groups.

Leukocyte ROS level measurement

To assess the ROS level in peritoneal leukocytes, flow cytometric analysis was performed (Van Pelt et al. 1996). Peritoneal exudates were centrifuged

(10 min, 300 g, 4˚C) and the collected leukocytes were diluted to a concentration $2x10^6$ cells/ml in PBS containing glucose (90 mg/100 ml PBS) and EDTA (20 mg/100 ml PBS). Cells were incubated at 37 ˚C for 45 min with the ROS-sensitive dye dichloro-dihydro-fluorescein diacetate (H_2DCFDA) at a concentration of 1μg/ml, and then placed on ice. The cellular fluorescence level was measured using a flow cytofluorimeter FACS Calibur (Becton Dickinson). To assess the level of autofluorescence, no H_2DCFDA was added to the control samples. The autofluorescence of leukocytes was very low and similar in all experimental groups so it was regarded as negligible.

The data recorded by the cytofluorimeter were analyzed using WinMDI version 2.8 software. Separate peritoneal leukocyte subpopulations were evident in density plots showing the size (FSC) and granularity (SSC) of the cells (Fig.1). To make certain that the correct subpopulations were being examined, macrophages and granulocytes were stained with monoclonal antibodies: Mac-1, Gr-1 (Farmingen, San Diego, CA at a concentration of $1\mu g/10^6$cells). The fluorescence (ROS) level of each population (geometric mean) was calculated using the WinMDI statistics function.

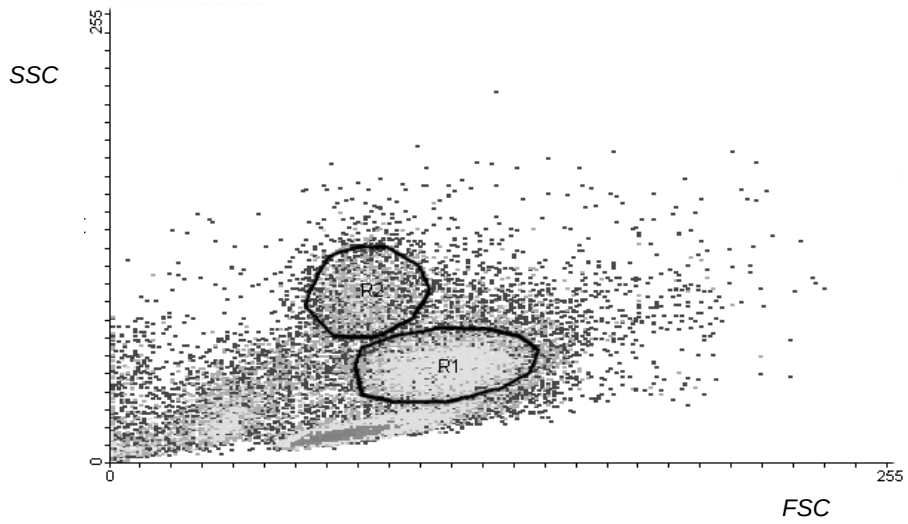

Fig. 1. **Density plot of peritoneal exudate cells from Zymosan-injected mice, showing size (FSC) and granularity (SSC)**. Clearly visible are the macrophage (R1) and granulocyte (R2) subpopulations. Leukocytes were stained with monoclonal antibodies to assign regions on the density plots to separate leukocyte subpopulations. Region 2 (R2), apart from granulocytes, also contains phagocytically-active, highly granular macrophages.

Splenocyte proliferation measurement

In vitro splenocyte cultures were set up according to a standard procedure (Drela and Zesko 2003).

Spleens were isolated aseptically, pooled within groups, gently homogenized with MEM (minimal Eagle's medium with L-glutamine and $NaHCO_3$) in a glass homogenizer and the homogenate filtered through nylon meshes to remove tissue debris. Cells were then collected by centrifugation (8 min, 310 g, 4˚C), resuspended in 4 ml of erythrocyte lysis buffer (0. 17 M Tris and 0.16 M NH_4Cl) and held at 4˚C for 15 min. After repeat centrifugation, the erythrocyte-free splenocytes were resuspended in a small volume and counted in a haemocytometer. Cell viability was estimated using the trypan blue exclusion test. Cells showing viability of ≥ 90%, were adjusted to a final concentration of 1 x 10^6 cells/ml with MEM medium supplemented with HEPES (20 mM), heat-inactivated foetal calf serum (10%), antibiotic-antimycotic (1%), sodium pyruvate (1 mM) and 2-mercaptoethanol (50 μM). A splenocyte culture (1×10^5 cells/well) was carried out in 96-well tissue culture plates (Falcon) in the presence of serial dilutions of PHA (0.25; 0.5; 1.0; 2.0 μg/100μl) or LPS (0.75; 1.5; 3.0; 6.0 μg/100μl). Control cultures consisted of cells incubated with the culture medium alone (spontaneous proliferation). Cell cultures were incubated for 72 h at 37°C in a fully humidified, 5% CO_2 atmosphere. Prior to harvesting with a semi-automatic cell harvester (Skatron), the cells were pulsed for 18 h with 1μCi/well of [^{3}H]-thymidine (UVVVR Prague, Czech Rep., 40 MBq/ml). Incorporation of tritiated thymidine was measured by liquid scintillation spectrometry (Beckman) and expressed as cpm and as the stimulation index, SI (cpm in mitogen-

stimulated culture divided by cpm in control culture).

Statistical analysis was performed using the parametric Student-Neuman-Keulus test at the significance level $2\alpha=0.05$.

RESULTS

The number of PTLs in mice from control, placebo-treated and TTO-inhaled groups was comparable, when they were left intact or injected with PBS. The sham-inhalation procedure did not influence an increase in the PTL number after Zymosan injection, while this increase was prevented by TTO inhalation treatment (the significant difference between TTO vs Intact group). The Zymosan-injected Placebo vs Zymosan-injected TTO was considered almost statistically significant (Fig. 2).

Naltrexone, an endogenous opioid receptor antagonist, given in drinking water, neither influenced the PTL number in both PBS-injected groups of mice (Placebo and TTO) nor modified the effect of Zymosan injection in the Placebo group. In contrast, it antagonized the inhibitory effect of TTO in Zymosan-treated mice (Fig. 3)

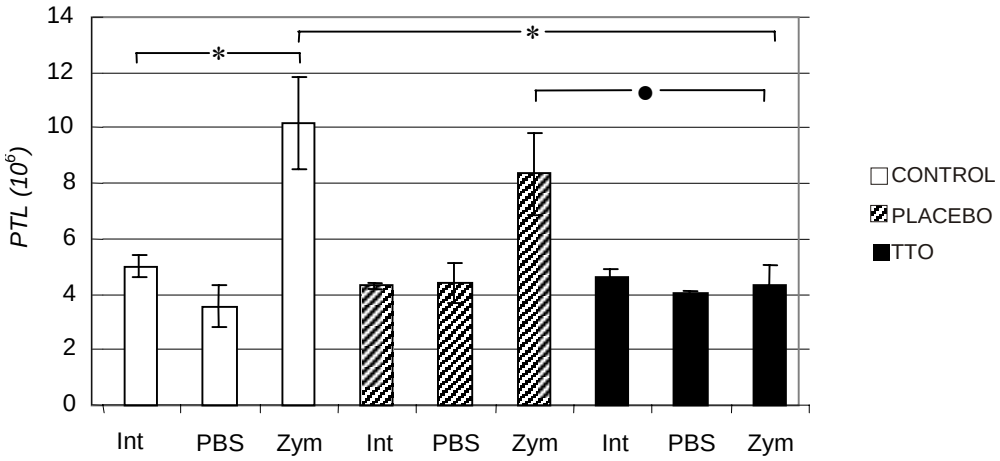

Fig.2. **Effect of TTO inhalation treatment on the number of peritoneal leukocytes in mice.** Statistical significance is shown as follows: * statistically significant; ● almost statistically significant (p=0. 056)

In both control and placebo-treated mice, an injection of Zymosan A caused the same result: a decrease in macrophage activity (Fig. 4A) accompanied by a simultaneous increase in granulocyte activity (Fig 4B). Both effects were reversed by the TTO inhalation treatment (Fig 4A and B).

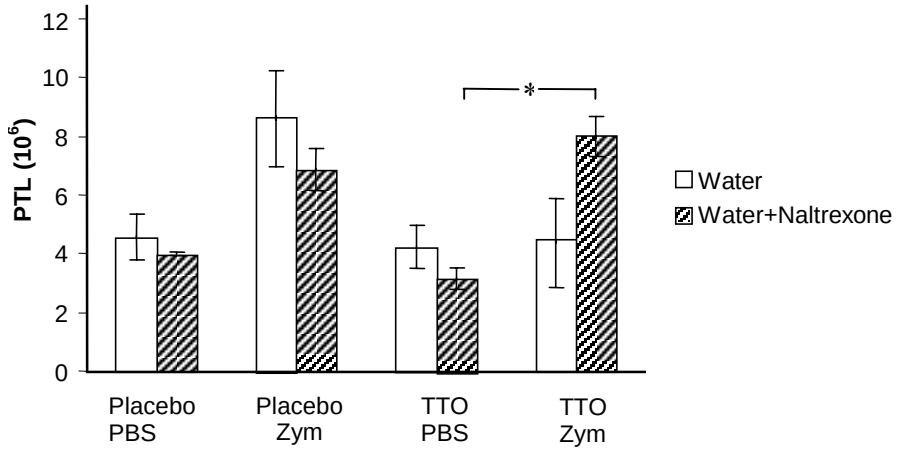

Fig. 3**. Modifications of peritoneal leukocyte number in mice by naltrexone administred in drinking water.**
***** statistically significant

A proliferation of splenocytes, isolated from the control (intact) mice was stimulated by both PHA (T-cell mitogen) and LPS (B-cell mitogen) in a concentration – dependent manner, reaching maximal SI values (near 4) when the mitogen concentrations were 1 and 0.75 μg/100 μl, respectively. A sham inhalation treatment (Placebo group) caused a very large increase in SI with maximal values exceeding 100 and 90, respectively, for the same mitogen concentrations. After the TTO

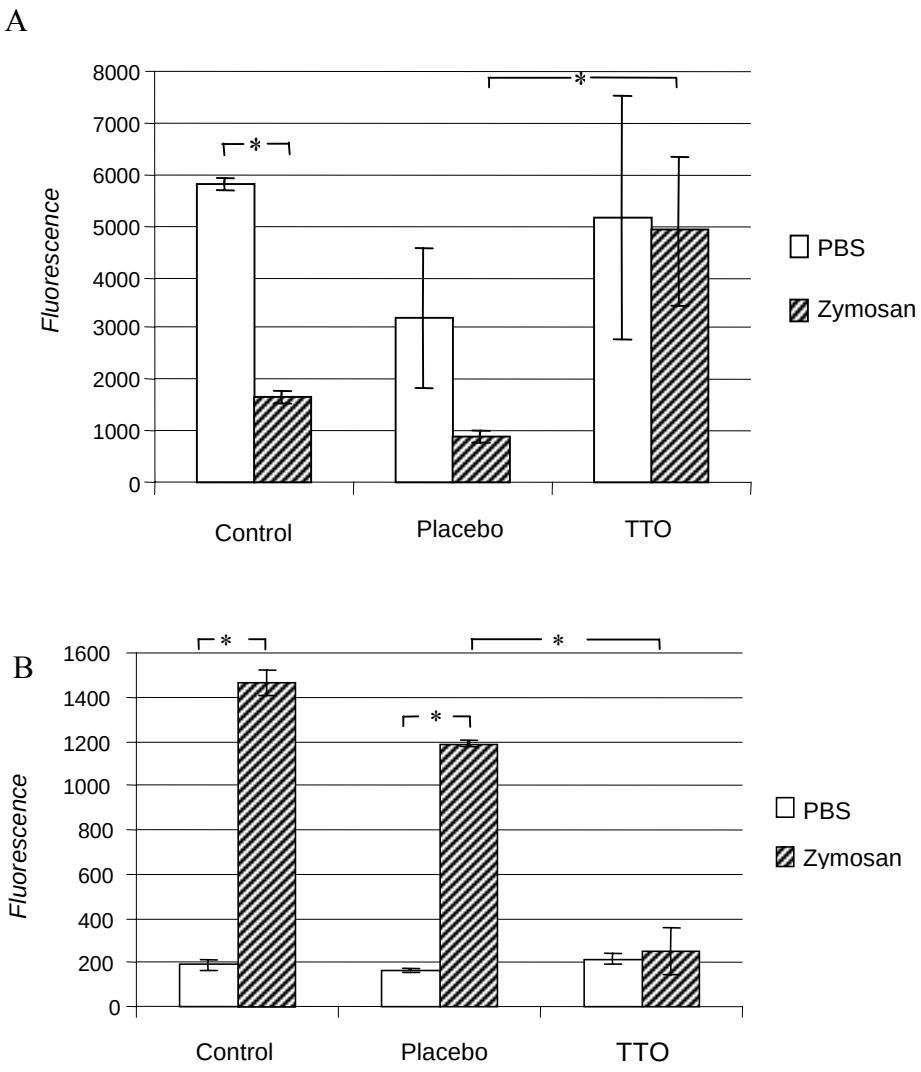

Fig. 4. **Effect of TTO inhalation treatment on the activity (ROS level) of macrophages (A) and granulocytes (B) in mice with experimental peritonitis.** * statistically significant

inhalation treatment splenocytes failed to respond to the same mitogenic stimulation (Fig. 5A and B).

DISCUSSION

The immunomodulatory action of TTO has been demonstrated by various experimental approaches but the mechanism of its influence on the immune system remains unknown. In the present study, the effect exerted by multiple sessions of TTO inhalation on an experimental inflammatory reaction (number of PTLs and their ROS level) as well as on splenocyte proliferation *in vitro* was examined.

The results obtained demonstrate the strong anti-inflammatory action of repeated TTO inhalation. In mice inhaling TTO three times a day for one week and then injected with Zymosan 24h before the end of the inhalation sessions, the number of PTLs was the same as in animals from the Control and Placebo groups without inflammation. This effect of TTO was completely abolished by naltrexone administered in drinking water, suggesting endogenous opioid involvement in the immunomodulatory action of inhaled TTO. Not only the peritoneal leukocyte number, but also

the activity of macrophages and granulocytes was restored by TTO to the level seen in the Control group without inflammation. In addition, mitogens of T and B-cells (PHA and LPS, respectively) at all concentrations used, had no stimulatory effect on splenocyte proliferation in the TTO treated group.

There appear to be several possible ways in which TTO can influence the immune system. A direct effect of TTO components on immune cells seems to occur when it is applied topically or dissolved in the circulating blood serum following inhalation. The essential oils are absorbed rapidly and become detectable in the blood serum after 15 min of inhalation (Kovar et al. 1987). The effects of such an action on immune cells has been observed in *in vitro* studies (Finlay-Jones et al. 2002a, b). Although TTO components can act directly on immune cells, naltrexone abolished the influence of inhaled TTO on the PTL number, suggesting some involvement of the nervous system and endogenous opioids in TTO inhalation-induced alterations of immune system functions. This is in agreement with published data demonstrating that inhaled essential oils cross the blood-brain barrier and influence central nervous system functions (Tisserand 1991, Buchbauer et al. 1993, Umezu 2002). TTO is believed to modify the synthesis and secretion of endogenous opioids, produced centrally and peripherally by the nervous system, which constitute an important set of chemical messengers connecting the nervous and immune systems (Peterson et al. 1998, Salzet 2001, Padget and Glaser 2003).

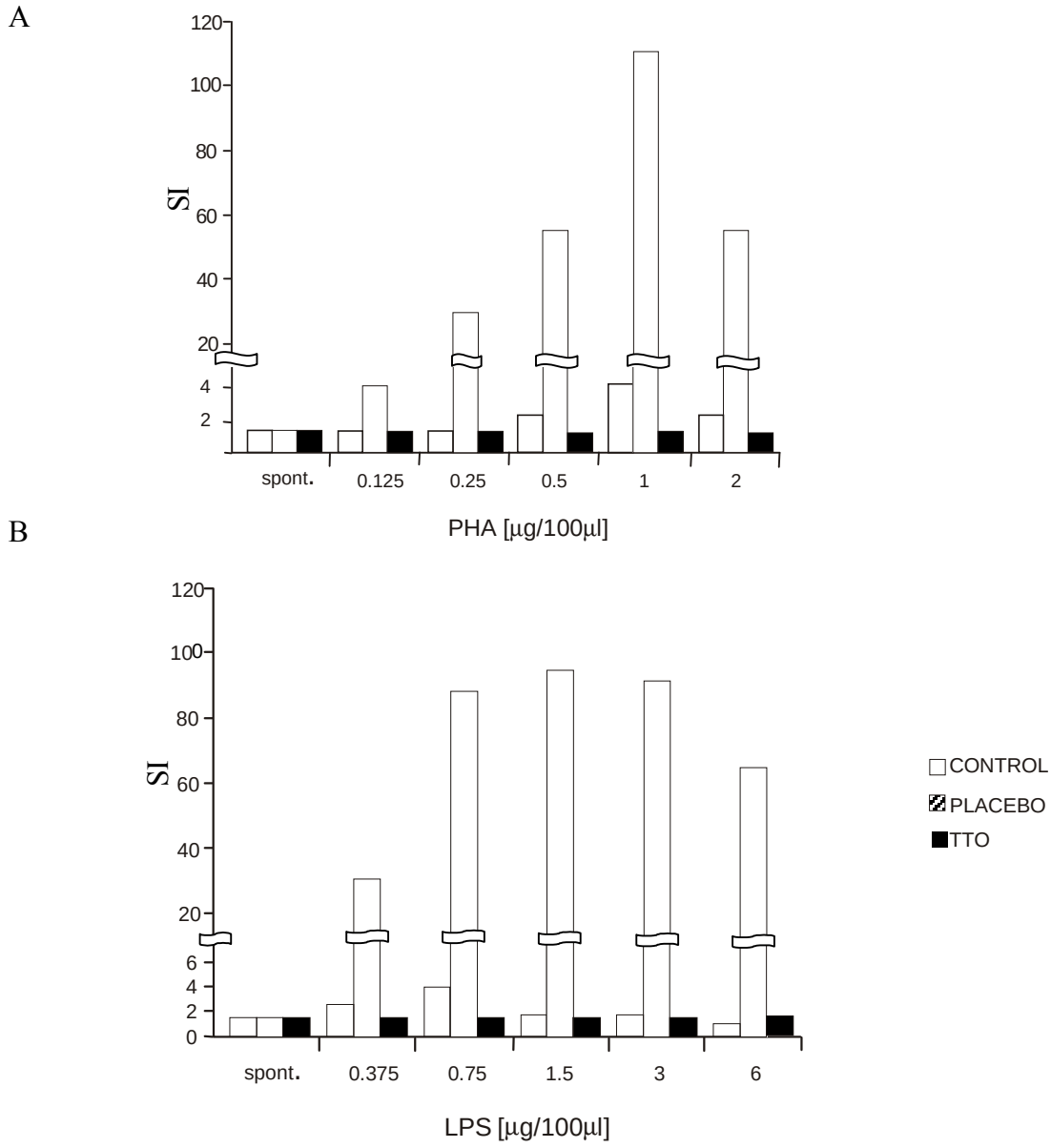

Fig. 5. **Mitogenic response of splenocytes from control, sham-inhalation and TTO inhalation group of mice.** Proliferation stimulated with various concentrations of PHA (A) and LPS (B), expressed as stimulation index (SI).

Another aspect of inhaled TTO action on the central nervous system is an anti-stress action (Alkiewicz 2000) which may, in turn, influence the immune system functioning. Other plant-derived essential oils are often used in anti-inflammatory therapy or as sedative agents. Their action on the immune system may be connected with the HPA axis (Moynihan et al. 1994, Fujiwara et al. 1998), endogenous opioids (Moynihan et al. 2000) or both of these mediator-systems.

The high level of PHA- and LPS-induced proliferation of splenocytes isolated from the Placebo group may be the result of stress occurring during the inhalation sessions, caused by handling and changes in the environment. Physiological or social stresses are known to influence the immune system functions e.g. increased stress was shown to enhance splenocyte responsiveness to mitogens in rats (Wood et al. 1993) and mice (Lyte et al. 1990). TTO inhalation treatment decreased splenocyte proliferation, probably by neutralizing the influence of stress on the immune system (effect of placebo inhalation). Further investigations are required to determine whether the action of inhaled TTO on immunity results from the modification of the HPA axis, nervous system and endogenous opioid changes or from the direct influence of TTO components on B and T-cells.

The results of this investigation demonstrate the strong anti-inflammatory activity of TTO, which in combination with its anti-microbial activity may give rise to successful therapeutic applications.

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REFERENCES

Ajuebor M.N. et al.: Endogenous monocyte chemoattractant protein-1 recruits monocytes in the zymosan peritonitis model. J. Leukoc. Biol. 63:108–116, 1998

Alkiewicz J., Kedzia B., Han S.: The role of tea tree oil in phytotherapy. Part II, Therapeutic application. Postępy fitoterapii 3/2000, (in Polish)

Brand C. et al.: Tea tree oil reduces swelling associated with the efferent phase of a contact hypersensitivity response. Inflam. Res. 51:236–244, 2002a

Brand C., Townley S.L., Finlay-Jones J.J., Hart P.H.: Tea tree oil reduces histamin-induced oedema in murine ears. Inflam. Res. 51:283–289, 2002b

Buchbauer G., Jirovets L., Jager W., Plank C., Dietrich H.: Fragrance compounds and essential oils with sedative effects upon inhalation. J. Pharm. Sci. 82:660–664, 1993

Carson C.F., Cookson B.D., Farrelly H.D., Riley T.V.: Susceptibility of methicillin-resistant *Staphylococcus aureus* to the essential oil of *Melaleuca alternifolia*. J. Antimicrob. Chemother. 35:421–424, 1995

Chadzinska M., Kolaczkowska E., Seljelid R., Plytycz B.: Morphine modulation of peritoneal inflammation in Atlantic salmon and CB6 mice. J. Leukoc. Biol. 65:590–596, 1999

Drela N., Zesko I.: Gender related early immune changes in mice exposed to airborne suspended matter. Immunopharm. Immunotox. 25:101–121, 2003

Finlay-Jones J., Hart P., Riley T.V., Carson C.: Terpinen-4-ol, the main component of the essential oil of *Melaleuca alternifolia* (TTO) suppresses inflammatory mediator production by activated human monocytes. In: Anti-inflammatory Activity of Tea Tree Oil, Rural Industries Research & Development Corporation Publication No 01/10, Chapter 2, 2002a

Finlay-Jones J., Hart P., Riley T.V., Carson C.: The water-soluble components of the essential oil of *Melaleuca alternifolia* suppress the production of superoxide by human monocytes, but not neutrophils, activated *in vitro*. In: Anti-inflammatory Activity of Tea Tree Oil, Rural Industries Research & Development Corporation Publication No 01/10, Chapter 3, 2002b

Fujiwara R. et al.: Effects of a long term inhalation of fragrances on the stress-induced immunosuppression in mice. Neuroimmunomodulation 5:318–322, 1998

Hammer K.A., Carson C.F., Riley T.V.: In vitro activity of *Melaleuca alternifolia* (tea tree) oil against dermatophytes and other filamentous fungi. J. Antimicrob. Chemother. 50:195–199, 2002.

Hildebrand J., Sheferd G.: Mechanism of olfactory discrimination: Converging Evidence for Common Principles Across Phyla. Annu. Rev. Neurosci. 29:595-631, 1997

Kolaczkowska E., Seljelid R., Plytycz B.: Critical role of mast cells in morphine-mediated impairment of zymosan-induced peritonitis in mice. Inflam. Res. 50:1–7, 2000

Komori T., Fujiwara R., Tanida M., Nomura J., Yokoyama M.M.: Effects of citrus fragrance on

immune function and depressive states. Neuroimmunomodulation 2:174–180, 1995

Kovar K.A., Kovar D.D., Johnson I.: 1,8 cineole was detected in the blood of mice following inhalation. Planta Med. 53:315–318, 1987

Lyte M., Nelson S.G., Baissa B.: Examination of the neuroendocrine basis for the social conflict-induced enhancement of immunity in mice, Physiol. Behav. 48:685–691, 1990

Majewski P., Markowska M., Laskowska H., Waloch M., Skwarlo-Sonta K.: Effect of morphine on thioglycollate-induced peritonitis in chickens. Neuroendocrinol. Lett. 23:87–93, 2002

Moynihan J.A., Karp J.D., Cohen N., Ader R.: Immune deviation following stress odor exposure: role of endogenous opioids. J. Neuroimmunol. 102:145–153, 2000

Moynihan J.A., Karp J.D., Cohen N., Cocke R.: Alterations in interleukin-4 and antibody production following pheromone exposure: role of glucocorticoids. J. Neuroimmunol. 54:51–58, 1994

Padgett D.A., Glaser R.: How stress influences the immune response. Trends Immunol. 24:444–448, 2003

Peterson P.K., Molitor T.W., Chao C.C.: The opioid-cytokine connection. J. Neuroimmunol. 83:63–69, 1998

Salzet M.: Neuroimmunology of opioids from invertebrates to human. Neuroendocrinol. Lett. 22:467–474, 2001

Schnitzler P., Schon K., Reichling J.: Antiviral activity of Australian tea tree oil and eucalyptus oil against herpes simplex virus in cell culture. Pharmazie. 56:343–347, 2001

Shnaubelt K.: Suppression of development of virus of influenza (*Myxovirus the influenzae*) under the inhalation of the TTO. Int. J. Aromather. 1:32, 1989

Skopinska-Rozewska E. et al.: Antimicrobial and immunotropic action of essential oils. In: Xenobiotics influence on the immune system, iss. IRS, Olsztyn, Poland, 127–136, 1997 (in Polish)

Tisserand R.: Essential oils as psychotherapeutic agents. In: Van Toller S., Dodd G.H.: Perfumery: The Physiology and Biology of Fragrance. London, England: Chapman & Hall, 167–185, 1991

Umezu T.: Pharmacological effects of plant-derived essential oils on the Central Nervous System. Aroma Research 3:376–382, 2002

Van Pelt L.J. et al.: Limitation on the use of dihydrorhodamine 123 for flow cytometric analysis of the neutrophil respiratory burst. J. Immunol. Methods 191:187–196, 1996

Wood P.G., Karol M.H., Kusnecov A.W., Rabin B.S.: Enhancement of antigen-specific humoral and cell-mediated immunity by electric footshock stress in rats. Brain Behav. Immun. 7:121–134, 1993