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Regulation of Immune Responses in Human Skin by Tea Tree Oil

**A report for the Rural Industries Research
and Development Corporation**

by John Finlay-Jones and Prue Hart

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Foreword

Studies in the laboratory and the clinic have in recent times confirmed the anecdotal anti-microbial properties of tea tree oil. On the basis of other anecdotal evidence it has been suggested that tea tree oil may have a valuable ability to treat a range of inflammatory conditions. Examples include insect bites and skin sensitivity reactions to a range of chemicals.

This report is presented by a research group which has previously established, in laboratory experiments, that tea tree oil can regulate the inflammation-related activities of human white blood cells. Further, they studied tea tree oil in inflammatory responses in an animal model. It limited several types of inflammation, with the timing of its application being an important factor in obtaining a significant outcome. Typically, application was most effective if made close to the time of onset of the inflammation.

The focus of this study shifts to humans. Tea tree oil was found to regulate the inflammatory response in humans given a small injection of histamine into skin (perhaps mimicking an insect bite). In a separate study, in humans who show skin sensitivity reactions to nickel, tea tree oil was found to lessen the sensitivity response, most noticeably in a subset of individuals. In summary, this report provides further evidence that tea tree oil has potential as an anti-inflammatory agent in humans. A remaining challenge is to determine why not all nickel-sensitive people respond well to tea tree oil.

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This report is an addition to RIRDC's diverse range of over 1000 research publications, forms part of our Tea Tree Oil R&D program, which aims to support the continued development of an environmentally sustainable and profitable Australian tea tree oil industry that has established international leadership in marketing, in value-adding, and in product reliability and production.

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Simon Hearn

Managing Director

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We are also grateful to Steen Jorsal of Novasel Australia Pty Ltd for his financial contribution to this research.

Abbreviations

CHS, contact hypersensitivity

EI, erythema index

FA, flare area

TTO, tea tree oil

Contents

Foreword	iii
Acknowledgments	iv
Abbreviations	iv
Executive Summary	vi
Chapter 1: Introduction	1
Chapter 2: Tea Tree Oil Reduces Histamine-Induced Skin Inflammation	3
2.1 Subjects and Methods	3
2.2 Results.....	4
2.3 Discussion.....	7
Chapter 3. Tea tree oil regulates nickel-induced contact hypersensitivity reactions in humans ..	9
3.1 Subjects and Methods	9
3.2 Results.....	12
3.3 Discussion.....	21
Chapter 4: References	24

Executive Summary

Tea tree oil is the essential oil steam-distilled from the Australian native plant, *Melaleuca alternifolia*, consisting of approximately 100 monoterpene and sesquiterpene hydrocarbons and alcohols. It is popular as a natural anti-microbial and anti-fungal therapeutic agent, with now many publications detailing the susceptibility of various microbes to tea tree oil. It has also been suggested that tea tree oil may have a valuable ability to treat a range of inflammatory conditions in skin. Examples include insect bites and skin sensitivity reactions to a range of chemicals, as well as infections. Until recently, there have been few scientific studies of the anti-inflammatory properties of tea tree oil.

Our initial studies, supported by the tea tree oil industry with RIRDC, were focussed on human white blood cells. These are the cells that infiltrate skin in response to infection, trauma or exposure to some chemicals. Neutrophils are the first white blood cells attracted into an inflammatory site. They can engulf and subsequently destroy foreign organisms. However, they are short-lived cells and at the site of infection their place is soon taken by monocytes and macrophages. Monocytes are present in blood and are also the precursors of macrophages in body organs and tissues. Monocytes and macrophages can produce a large range of potent chemicals which are responsible for an inflammatory response and the associated tissue damage.

These earlier studies showed that, in laboratory-based experiments, tea tree oil and selected pure components could regulate the way some of these white blood cell types, and not others, responded to challenge. One response of neutrophils to infection is to activate pathways that kill the germs they have engulfed. Tea tree oil did not affect these pathways. In contrast, monocytes were significantly affected. Their ability to release chemical mediators that promote inflammation was inhibited by tea tree oil. The results supported the use of tea tree oil in infection, in that the oil would not impair the ability of neutrophils to kill germs. They also indicated a potential role as an anti-inflammatory agent, by being able to regulate the cells which promote inflammation and damage living tissue.

There is considerable anecdotal evidence that tea tree oil may have beneficial effects on the itching of inflamed skin (associated with insect bites and some chemicals). Also, one clinical study of tea tree oil cream as an antifungal agent had noted that it improved the inflammatory symptoms (itch, burning and scaling) associated with tinea pedis (“athlete’s foot”). These observations plus the results of our experiments on human white blood cells formed the basis for subsequent studies, in which the focus moved from laboratory-based experiments to models of inflammatory responses in animals.

Two types of skin “hypersensitivity” in mice were looked at. The first was a model of “delayed” or “contact” hypersensitivity, one example of which is the sensitivity that up to one in ten humans, especially females, have to nickel (such as found in some jewellery, and jeans studs). A second example is the sensitivity to poison ivy found in North America. In the mouse model, the chemical trinitrochlorobenzene is used to initiate the hypersensitivity. The results showed that tea tree oil applied 30 minutes before or up to 7 hours after chemical challenge reduced the swelling associated with the inflammatory response in mouse skin.

This investigation also asked the question whether any of the major, purified components of tea tree oil could affect the swelling associated with a contact hypersensitivity response. The results suggested that terpinen-4-ol (approximately 40% of tea tree oil) and α -terpineol (approximately 3% of tea tree oil) were principally responsible.

The second model studied involved “immediate-type” hypersensitivity, one type of allergy, examples of which in humans include skin hives and the potentially life-threatening effects of bee stings in some individuals. In this type of hypersensitivity, cells in the skin known as mast cells release a potent chemical, histamine, which is responsible for many of the symptoms of these hypersensitivity

responses. The effects of histamine can be seen within minutes of the body's exposure to the allergy-inducing substance.

The studies undertaken in mice looked at the effect of tea tree oil on the swelling in skin that followed histamine injection. Tea tree oil reduced swelling only if applied immediately after histamine injection. Terpinen-4-ol, the major water-soluble component of tea tree oil, was shown to be equivalent in potency to whole tea tree oil in the reduction of histamine-induced ear swelling.

In contrast, no beneficial effect was found for tea tree oil in a model of mild sunburn in mice.

In the present study we build on these experimental models with a study of the effect of tea tree oil on skin responses in two different circumstances in humans.

The first focused on the response of human skin to a small injection of the chemical histamine. This is a chemical that is released by cells in the skin (known as mast cells). The effects of histamine include the stimulation of blood vessels to dilate, which shows as redness (a "flare"), and to release fluid into the skin, which causes swelling (a "wheal"). These responses can be seen, for example, in some insect bites, so histamine injection is one way of mimicking this.

Small amounts of histamine were injected into the skin in each forearm of 27 volunteers. Most (21) of the volunteers were then treated with tea tree oil at one of the injection sites after 20 minutes, with some (6) having paraffin oil applied. The redness and swelling at both the treated and untreated injection sites were measured every 10 minutes for a period of an hour. Application of liquid paraffin had no significant effect on histamine-induced wheal and flare. In contrast, whilst tea tree oil did not affect the development of the redness, the swelling was significantly reduced. This study was repeated with an additional 15 volunteers, with tea tree oil being applied at 10 then 20 minutes, which resulted in both the redness and the swelling being lessened by the oil.

This is the first study to show that tea tree oil can reduce experimentally-induced inflammation in human skin and gives experimental support to anecdotal reports that tea tree oil can reduce the hypersensitivity responses to insect bites.

The second study investigated whether tea tree oil could modulate a recall immune response in humans as it does in mice (recall immune responses are the basis of contact hypersensitivities, or more generally, delayed hypersensitivities). Large numbers of people, especially females, are hypersensitive to nickel. We examined whether tea tree oil could reduce a nickel-induced rash in these subjects.

Eighteen nickel-sensitive subjects were evaluated for the effect of tea tree oil on a nickel-induced rash. Small amounts of nickel were applied to the back skin. Subsequently, either 100% tea tree oil, 5% tea tree oil lotion, a placebo lotion (no tea tree oil), or 100% macadamia oil was applied at days 3 and 5 after nickel exposure. Anti-inflammatory effects were found with 100% tea tree oil but were predominantly, although not exclusively, seen in a subgroup of nickel-sensitive subjects with a prolonged development phase of nickel-induced CHS. These comprised about one third of total patients. The 5% tea tree oil lotion, placebo lotion and the 100% macadamia oil were all without significant effect.

In order to explore a potential mechanism of action for tea tree oil, we studied the response of white blood cells from these patients to exposure to nickel in laboratory studies. A typical response was to proliferate, which correlates with the development of a rash. Tea tree oil added to these stimulated white blood cells inhibited their proliferation, consistent with an ability to inhibit the development of a rash. Importantly, tea tree oil was not toxic for the cells at the dose that inhibited proliferation.

On the basis of these results, we can conclude that topical application of 100% tea tree oil may be useful in treating nickel-induced rash in the skin of human sensitive to the chemical. Whether this is the case for other mild allergies and sensitivities, and why it is that some people appear to respond better than others, need investigation.

In summary, our results suggest that tea tree oil can be used for the treatment of inflammatory reactions of the skin including those following insect bites and exposure to nickel, and it may be possible to extend that treatment to sensitivity reaction to other chemicals including plant components and other irritants.

Chapter 1: Introduction

Tea tree oil (TTO) is the essential oil steam-distilled from *Melaleuca alternifolia*, an Australian native plant. TTO contains over 100 components, the majority being monoterpene and sesquiterpene hydrocarbons and their alcohols. Several *in vitro* studies have investigated TTO's antimicrobial properties and there are now susceptibility data on a wide range of bacteria, yeasts and fungi.¹⁻⁴ In recent years, TTO has become popular as a naturally occurring antimicrobial and antiseptic agent.

A recent systematic review of randomised clinical trials with TTO for treatment of acne and fungal infections concluded that due to promising findings, TTO deserves to be investigated more closely.⁵ Some clinical and *in vitro* studies have reported indirect anti-inflammatory responses with topical TTO.^{1,4,6-8} We hypothesise that these anti-inflammatory responses may reflect control of the tissue damaging effects of a strong immune response induced by an invading organism. We have reported regulatory properties of TTO on the activity of human monocytes activated *in vitro*.^{9,10} In contrast, TTO could not control superoxide production by human neutrophils *in vitro*.¹⁰ However, monocytes, macrophages and neutrophils may not be part of the immediate hypersensitivity response to allergens or components of an insect bite, a condition that is also treated with TTO. As allergen-induced wheal and flare responses are mediated mainly by histamine¹¹, we tested topical TTO on experimentally-induced skin inflammation induced by histamine. In an experimental model in mice, we demonstrated that TTO could lessen the swelling induced in the ear by a small injection of histamine.¹² We then examined the ability of TTO to lessen the redness and swelling that followed injection of a small amount of histamine in human skin. This study is described in Chapter 2 of this report.

In addition to testing the ability of TTO to treat the redness and swelling that results from the release of histamine in the skin following for example an insect bite, we have evaluated its ability to lessen the skin reactions that can follow exposure of some individuals to certain chemicals to which they are sensitive.

In mice, TTO added prior to, or up to 7 hours after administration of the sensitizing antigen, inhibited the inflammatory component of a contact hypersensitivity (CHS) response (an immunological response involving memory T lymphocytes) to a chemical hapten.¹³ However, TTO did not affect the inflammatory response to the non-immunological irritant stimulus, UV radiation. Therefore, TTO significantly suppressed a systemic recall immune response.

Nickel-induced CHS is the commonest cause of occupational contact dermatitis in humans.¹⁴ The overall incidence in the Caucasian population is up to 20 percent, with females being affected more than males.¹⁵ For those who have a severe allergy, tasks such as handling coins and doorknobs can become problematic. For most people, complete avoidance of nickel-containing metals and foods is impossible or impractical. Some patients choose to tolerate the skin inflammation and irritation that occurs after contact with nickel-containing objects such as metal belt buckles and cosmetic jewellery. Depending on the duration of contact, the concentration of nickel, and the level of nickel-sensitivity, the skin irritation can develop into blistering lesions that may become secondarily infected.

Nickel-induced allergic contact dermatitis in nickel-sensitive subjects is a novel method of studying inflammation associated with delayed-type hypersensitivities in humans. This experimentation does not require prior sensitisation of volunteers to a chemical hapten (e.g. diphenylcyclopropanone) as occurs in some studies.¹⁶

The study presented in Chapter 3 of this report investigated whether TTO could modulate a recall immune response in humans to nickel. The potential of TTO to regulate a nickel-induced delayed type hypersensitivity reaction when applied after nickel exposure was investigated. In addition to 100% TTO, the effect of a 5% TTO lotion was investigated and compared with the action of a placebo lotion of the same formulation minus the TTO. Refined macadamia oil was used as a control for the TTO.

Chapter 2: Tea Tree Oil Reduces Histamine-Induced Skin Inflammation

2.1 Subjects and Methods

Participants

In the group treated with the control oil, liquid paraffin, there were 5 females and 1 male, mean age 37, range 23-54 years. Twenty-one people were tested with a single application of TTO (16 females, 5 males, mean age 35, range 23-56 years). Participants had no severe generalized skin conditions such as eczema or psoriasis, atopy (eczema, hayfever or asthma), or previous skin or systemic sensitivity to TTO and had had no severe allergic reactions in the past. Subjects with a past history of pityriasis versicolor, tinea pedis, minor acne and minor scalp psoriasis were included in the study. The participants were not on systemic immunosuppressant therapy and had not taken oral anti-histamines or topical corticosteroids in the preceding 2 weeks. This study was approved by the Clinical Investigation Committee of Flinders Medical Centre, Adelaide, Australia. A second study undertaken with a group of 15 volunteers examined the effects of two applications of TTO.

Induction of wheal and flare

Histamine (50 µl of 100 µg/ml solution) was injected intradermally into the inner forearm skin (approximately midway along the volar aspect) of both arms and the resulting wheal and flare measured at 10 min intervals for 60 min. After 20 min, undiluted TTO (25 µl) or liquid paraffin (25 µl) was applied topically with a pipette to cover the flare and wheal on the experimental arm. Study arms (TTO or liquid paraffin) and control arms were assigned in an alternating fashion from subject to subject. In this way each subject acted as his or her own control. Wheal and flare diameters (cm) were measured with calipers (Mitutoyo Corp., Tokyo, Japan). Wheal skin double thickness (mm) was measured by lightly pinching the skin and measuring with a spring-loaded gauge (Mitutoyo). In the second study, TTO was applied at 10 and 20 min after histamine injection.

Flare area was calculated by using the following formula.¹⁷

$$\text{Flare area (cm}^2\text{)} = \pi/4 \times (D_1 + D_2)^2/2$$

Where D_1 = diameter of flare (cm)
 D_2 = second perpendicular diameter of flare (cm)

Assessment of wheal volume was calculated using the following formula.¹⁷

$$\text{Wheal volume (}\mu\text{l)} = \pi/4 \times (d_1 + d_2)^2 /2 \times (T_t - T_0) /2$$

where d_1 = diameter of wheal (cm)
 d_2 = second perpendicular diameter of wheal (cm)
 T_t = skinfold thickness at time t (mm)
 T_0 = skinfold thickness at time 0 (mm)

Subjects were also questioned about level of itch during the experiment and asked to grade pruritus 0=no itch, 1=mild, 2=moderate and 3=severe. They were also asked if they had previously used TTO products.

Tea Tree Oil and Liquid Paraffin

The TTO was provided by Thursday Plantation (Ballina, NSW, Australia) as is commercially available. Gas chromatographic analysis of the TTO used in this study by Wollongbar Agricultural Institute, Wollongbar, Australia, showed the following concentrations, terpinen-4-ol 41.6%, γ -terpinene 21.5%, α -terpinene 10.0%, terpinolene 3.5%. α -terpineol 3.1%, α -pinene 2.4%, 1,8-cineole 2.0%, p-cymene 1.8%, aromadendrene 1.1%, δ -cadinene 1.0%, limonene 0.9%, ledene 0.9%, globulol 0.5%, sabinene 0.4% and viridiflorol 0.2%. TTO was kept in 10 ml aliquots (brown glass bottles) to minimise oxidation and discarded after 1 month. Liquid Paraffin (B.P.) was obtained from Orion Laboratories (Welshpool, Western Australia).

2.2 Results

All of the subjects tolerated intradermal histamine injection and topical application of TTO or liquid paraffin without any adverse effects. Of the 21 subjects in the TTO study, 7 had not used any TTO product before on the skin. The other 14 subjects had each used one or more of a range of products with unknown concentrations of TTO (e.g. cream, deodorant, moisturizer, soap, handwash) as well as 100% pure oil, on limbs or face between 1 week to 1 year preceding the study. Stated uses for the products were for insect bites, cuts, acne or skin irritation.

There was no significant difference in itch scores between the control, TTO- and liquid paraffin oil-treated arms. The control oil, liquid paraffin, had no effect on mean flare area over the 60 min period following histamine injection (Fig 1A). There was also no significant difference in mean flare area between control and TTO arms over the same period following histamine injection (Fig 1B).

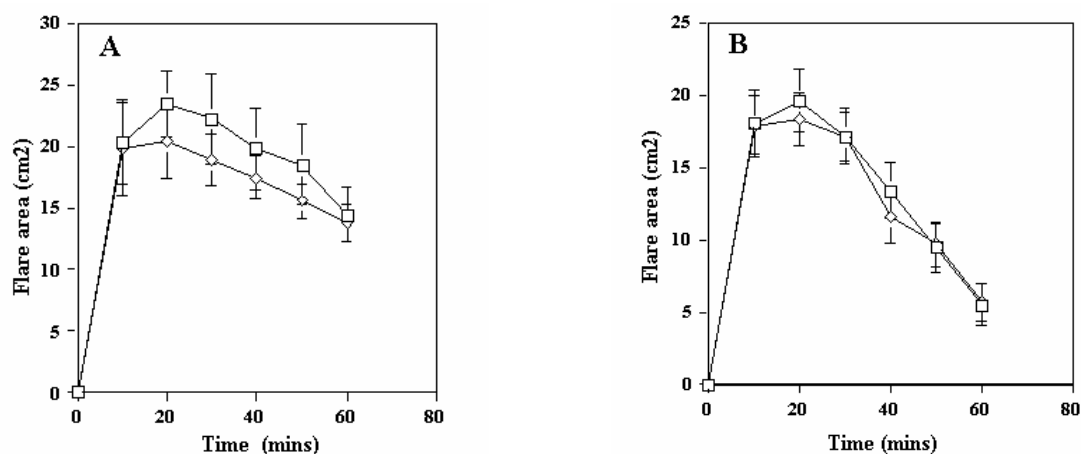


Figure 1. The effect of A. liquid paraffin, and B. tea tree oil on histamine-induced flare. The mean flare area (cm²) for the control (solid line) and study arms (broken line) with increasing time after histamine injection is shown. In A. liquid paraffin, and in B. tea tree oil, was applied 20 min after histamine administration. The mean result for A. 6 volunteers \pm SEM, and B. 21 volunteers \pm SEM, is shown.

Table 1. Wheal volumes (μl) 20, 40 and 60 min after histamine injection for 28 volunteers treated after 20 min with liquid paraffin (LP) or tea tree oil (TTO).

Subject	Time (min)	Control Arm μl	Study Arm μl	Subject	Time (min)	Control Arm μl	Study Arm μl
LP1	20	8.04	10.9	LP4	20	1.13	2.33
	40	5.45	10.34		40	2.70	2.94
	60	5.31	9.08		60	1.25	1.25
LP2	20	4.32	4.18	LP5	20	1.81	0.93
	40	2.84	5.31		40	1.50	0.67
	60	1.94	4.01		60	1.50	0.08
LP3	20	3.68	4.38	LP6	20	0.67	2.68
	40	2.68	1.42		40	1.35	3.85
	60	2.19	0.53		60	1.11	2.70
TTO1	20	1.58	1.70	TTO12	20	2.39	2.65
	40	1.58	0.78		40	2.76	2.86
	60	1.13	0.73		60	1.47	2.76
TTO2	20	2.15	3.28	TTO13	20	0.66	0.66
	40	3.72	3.31		40	2.65	1.43
	60	2.04	2.80		60	2.31	0.00
TTO3	20	0.26	0.69	TTO14	20	0.61	1.70
	40	1.96	0.76		40	1.99	1.47
	60	1.25	0.90		60	1.35	1.36
TTO4	20	2.12	5.94	TTO15	20	1.33	2.33
	40	1.85	2.86		40	1.43	2.33
	60	0.53	1.58		60	1.56	0.99
TTO5	20	0.53	1.41	TTO16	20	0.43	0.57
	40	0.43	1.41		40	0.48	0.31
	60	0.25	0.00		60	0.09	0.06
TTO6	20	1.45	1.10	TTO17	20	1.38	1.99
	40	1.72	0.62		40	2.65	1.46
	60	0.52	0.98		60	1.33	0.21
TTO7	20	2.60	2.26	TTO18	20	1.72	1.13
	40	1.69	2.15		40	0.90	0.52
	60	1.70	0.23		60	0.00	0.00
TTO8	20	0.35	2.38	TTO19	20	1.28	1.23
	40	0.40	2.86		40	1.84	0.95
	60	0.24	1.85		60	0.39	0.00
TTO9	20	1.33	0.66	TTO20	20	2.00	1.23
	40	1.69	0.14		40	2.48	0.88
	60	1.72	0.12		60	1.99	0.83
TTO10	20	2.08	1.43	TTO21	20	1.28	0.95
	40	2.07	1.77		40	2.58	0.08
	60	0.80	0.94		60	2.28	0.08
TTO11	20	0.54	2.26				
	40	1.36	1.30				
	60	0.95	0.90				

The mean wheal size 20 min after histamine injection was 2.05 μl , range 0.26 – 10.90 μl (Table 1). As there was considerable inter-individual, as well as intra-individual variability in the size of the histamine-induced wheal, the results were normalized as a percentage of the wheal volume at 20 min.

For the 6 volunteers treated with liquid paraffin twenty min after histamine injection, there was no significant difference in the wheal between the control and study arms (Figure 2A). It is notable that the wheal volume continued to increase after application of liquid paraffin. In contrast, the mean wheal volume showed a marked decrease following TTO application 20 min after histamine injection (Fig 2B). At 30 min (10 min after TTO application), the wheal had increased in size in only 6 of the 21 arms treated with TTO. This contrasted with an increased wheal in 17 of the 21 untreated control arms. At 30 min (10 min after TTO application), the mean wheal volume on the TTO arm was 92% of that seen at 20 min. It decreased to 83%, 62% and 43% at 40, 50 and 60 min, respectively. At 30 min, the mean wheal volume on the control arm was 163% of that seen at 20 min, changing to 175%, 130%, and 113% at 40, 50 and 60 min respectively (Fig 2B). At 30 min, the % wheal volume of the TTO-treated arms was statistically significantly lower than that of the control arms ($p=0.0004$, Mann Whitney U). At 60 min, the % wheal volume of the TTO-treated arms was also statistically significantly lower than that of the control arms ($p=0.017$).

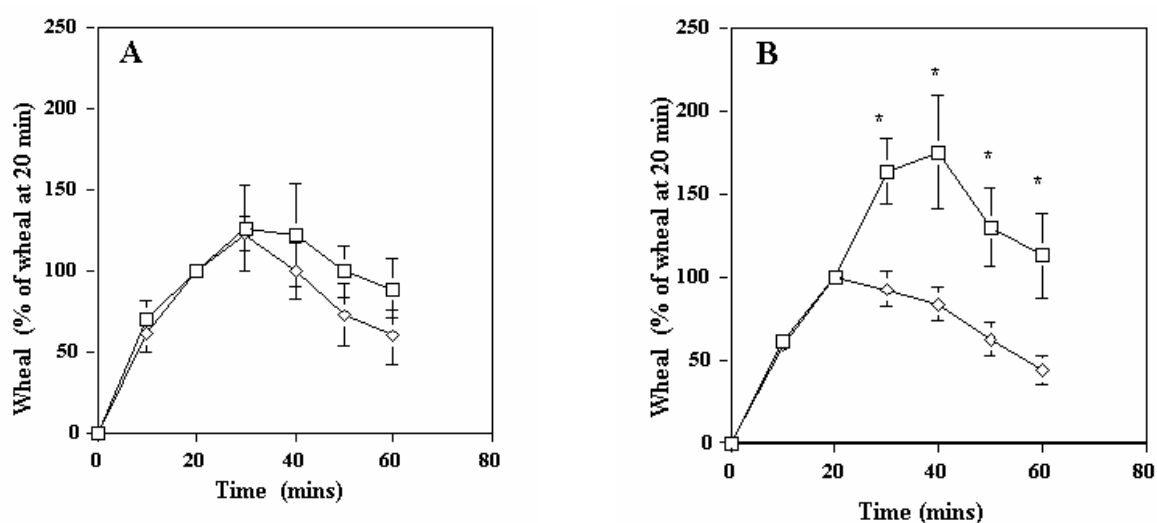


Figure 2. The effect of A. liquid paraffin, and B. tea tree oil, on histamine-induced wheal.

The wheal volume of the control and study arms 20 min after histamine injection (and time of application of liquid paraffin or tea tree oil) was calculated as 100%. The mean percentage change in wheal volume for the control (solid line) and study arms (broken line) with increasing time after histamine injection is shown. The mean result for A. 6 volunteers \pm SEM, and B. 21 volunteers \pm SEM, is shown. An asterisk indicates a significant difference between control and TTO-treated arms.

In a second study involving 15 volunteers, TTO was applied at 10 and 20 min after the histamine injection. As was the case for the first study, TTO application inhibited the histamine-induced wheal, and it also significantly inhibited the histamine-induced flare at 40 to 60 minutes (Figure 3).

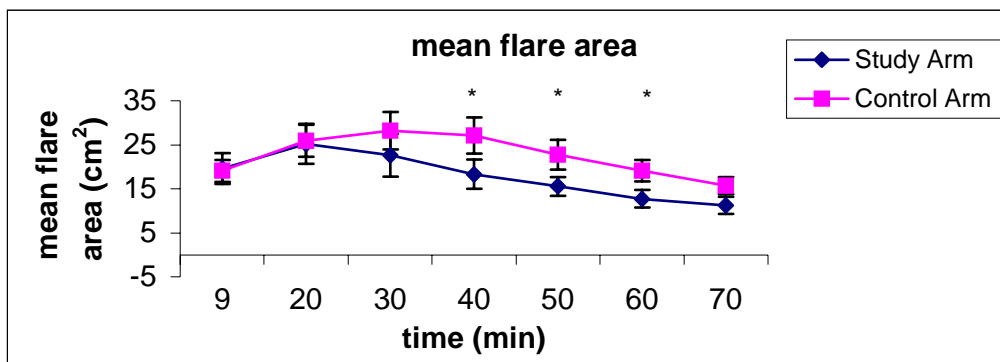


Figure 3. The effect of tea tree oil on histamine-induced flare.

The mean flare area (cm²) for the control (squares) and study arms (diamonds) with increasing time after histamine injection is shown. Tea tree oil was applied at 10 and 20 min after histamine administration. The mean result \pm SEM for 15 volunteers is shown.

2.3 Discussion

In this study, a wheal and flare reaction of significant size was induced by histamine injection in the inner forearms of 28 volunteers. There was considerable inter-individual and intra-individual variability in the response to histamine. The intra-individual variability between one's arms was surprising as the control and liquid paraffin- and TTO-treated arms were used alternatively. For this reason, the wheal results were normalized to that measured at 20 min, i.e. the wheal measured immediately before application of liquid paraffin or TTO. TTO significantly reduced the developing oedema to histamine whilst the wheal in the liquid paraffin-treated and control arms continued to develop. Twenty minutes was chosen as the time for application of TTO or the control oil as we hypothesized that this was similar to the timing of medication after an insect bite or allergen exposure. In this study, we used liquid paraffin as a control oil. Furthermore, it was unable to reduce histamine-induced wheal and flare in human skin. No oil could be considered the ideal control oil for TTO. Although less volatile, liquid paraffin was considered the best example of an immunologically inert oil.

Histamine-induced inflammation of the skin is manifest by initial reddening of the skin, plasma extravasation and the development of a wheal (tissue oedema) and a flare (wider spread erythema). This reaction is frequently accompanied by pruritus (itch). Local release of vasoactive substances from sensory nerves, the vascular endothelium and infiltrating blood cells mediate these changes in microvascular perfusion and permeability; TTO may be affecting any one of these mechanisms of oedema formation. Histamine-induced inflammation is most often associated with immediate hypersensitivity reactions, with histamine released from mast cell granules.

This laboratory has shown that the water-soluble components of TTO, especially terpinen-4-ol which comprises 40% TTO, can suppress inflammatory mediator production by activated human monocytes.⁹ The production of lipopolysaccharide-induced tumour necrosis factor- α , often considered the most influential inflammatory cytokine,¹⁸ as well as IL-1 β , IL-8, IL-10 and prostaglandin E₂, was suppressed. Also, the water-soluble components of TTO can suppress the production of superoxide by human monocytes, but not neutrophils, activated *in vitro*.¹⁰ TTO may enable neutrophils to be fully active in an acute inflammatory response and eliminate foreign antigens, while suppressing monocyte production of superoxide and inflammatory mediators thereby preventing oxidative damage and the activation of other cells that is seen in more chronic inflammatory states.

Antimicrobial susceptibility studies have found TTO effective *in vitro*.^{1,4,7,8} TTO has also been trialled as a pediculocide with 100% mortality of adult head lice.¹⁹ However, few clinical studies have tested TTO's antimicrobial or anti-inflammatory effects *in vivo*. In one study⁷, TTO cream improved the symptoms of tinea pedis (i.e. scaling, inflammation, itch, burning) compared to placebo, with excellent skin tolerance. Interestingly there was no statistically significant difference in fungal clearance between the two groups. In another study⁶, 5% TTO in a water-based gel was an effective topical treatment for acne vulgaris, although less effective than a 5% benzoyl peroxide water-based lotion because of its slower onset of action. The TTO formulation was better tolerated on facial skin, with less skin scaling, dryness and pruritus than with benzoyl peroxide.

In recent years there has been increasing interest in “natural” medicine products with a special demand for Australian TTO. This study shows that undiluted TTO applied to histamine-induced inflammation can reduce mean wheal volume and flare area. This is the first study to show that TTO can reduce experimentally-induced inflammation in human skin and may give some credence to anecdotal reports that TTO can reduce the hypersensitivity responses to insect allergens. The mechanism by which the active ingredients of TTO regulate wheal formation (or fluid resorption) is as yet unknown.

Chapter 3. Tea tree oil regulates nickel-induced contact hypersensitivity reactions in humans

3.1 Subjects and Methods

Subjects

Eighteen subjects with nickel hypersensitivity were studied for regulation by TTO of their CHS response to nickel. The study was approved and performed in accordance with the guidelines of the ethics committee at Flinders Medical Centre, and all subjects gave their informed consent to participation. Subjects self-referred from advertisements in a local newspaper and a radio station. Seventeen of these patients were female (mean age 37 years, range 19 to 57 years), and one was male (age 21 years).

Peripheral blood was collected from 11 nickel-sensitive subjects and 6 control subjects in order to investigate the effect of TTO on the proliferative response of mononuclear cells. All subjects were female (mean age 42 years, range 19 to 67 years). Ten of the nickel-sensitive subjects had participated in the study investigating the effect of TTO on nickel-induced hypersensitivity reactions and had therefore been confirmed as nickel allergic by patch testing. One nickel-sensitive subject required patch testing (see below) to nickel prior to venesection to confirm nickel hypersensitivity. Six healthy female subjects, aged between 21 and 50 years (mean age 37 years), participated as control subjects.

All subjects completed a questionnaire relating to their history of nickel sensitivity and relevant past medical history. Exclusion criteria included age younger than 18 years, pregnancy or breast-feeding, immunosuppression due to illness or medication, or history of extreme nickel sensitivity.

Tea tree oil

TTO was kindly supplied by Novasel Australia Pty Ltd (Mudgeeraba, Qld, Australia) and fulfilled the criteria of the Australian Standard²⁰ with a terpinen-4-ol level greater than 30% and 1,8-cineole less than 15% as determined by gas chromatography-mass spectrometry. The 5% TTO lotion and a placebo lotion (no TTO) (Novasel) contained 10% olive oil PEG 7 esters, 1% vitamin E, 5% Aloe Vera Gel, 1% Benzyl alcohol, and water.

The water-soluble components of TTO were obtained as previously described.^{9,10} Briefly, TTO preparations of 1.25% (v/v) were prepared in polystyrene plastic tubes in serum-free culture medium, mixed well and left to stand for 30 min. Gas chromatography/mass spectrometry confirmed that the water-insoluble components adhered to the side of the plastic tubes whilst the water-soluble components remained in the culture medium.⁹

Triple filtered, refined macadamia oil (Bronson and Jacobs Pty Ltd, Homebush Bay, NSW, Australia) contained approximately 80% monosaturated acids in its triglycerides, of which 15-21% was palmitoleic acid.

Testing of nickel sensitivity

To confirm nickel hypersensitivity, subjects were patch tested with three different concentrations of nickel depending on their history of nickel sensitivity.

Nickel sulphate (5%) in petrolatum (Hermal-Trolab, Reinbek, Germany) was diluted in petrolatum to concentrations of 2.5%, 2%, 1%, 0.5%, 0.25%, 0.125%, 0.06%, and 0.03% by the pharmacy at Flinders Medical Centre. All nickel sulphate preparations were stored in the dark at 4°C.

Nickel was placed in 9 mm finn chambers (Epitest, Finland) on tape (Scanpor, Norgesplaster, Norway) on the lower back under waterproof dressings (Opsite, Smith and Nephew, Victoria, Australia). The patches were removed on day 3 and readings of erythema were made one hour later. The concentration of nickel chosen for later study was the concentration associated with moderate confluent erythema. In the study, 5 patients required 5% NiSO₄ to achieve a moderate confluent erythema, 1 required 2%, and 2, 4, 2, 3, and 1 required 1%, 0.5%, 0.25%, 0.125%, and 0.06% NiSO₄, respectively.

Regulation by TTO of CHS response to nickel

For the 18 subjects, nickel sulphate (at the concentration required to induce a moderate confluent erythema) was placed in 9 mm finn chambers on tape (Scanpor) and placed on the upper back in quadrants (Table 1). The patches were left in place and covered with waterproof dressings until removal on day 3. The subjects were asked to refrain from excessive physical activity causing sweating or from swimming during the study.

Table 1. Treatment protocols for nickel-induced CHS sites.

<u>PROTOCOL 1^a</u>		<u>PROTOCOL 2^a</u>	
Ni alone	Ni + TTO	Ni alone	Ni + TTO
Ni + placebo lotion	Ni + TTO lotion	Ni + macadamia oil	Ni alone
	Ni + placebo lotion	Ni + TTO	
<i>control placebo only^b</i>	Ni + TTO	<i>control TTO only^b</i>	Ni + macadamia oil
<i>control TTO lotion only^b</i>	Ni alone	<i>control macadamia only^b</i>	Ni alone
<i>control TTO only^b</i>	Ni + TTO lotion		Ni + TTO

^a An area of 300 cm² on the back skin of each subject was divided into quadrants.

Up to 8 sites (as indicated) were challenged with nickel. On day 3, additional treatments were applied as indicated (25 µL of either 100% TTO, 5% TTO lotion, placebo lotion, or 100% macadamia oil).

^b Sites without nickel were tested as single tests and were control sites for 100% TTO, 5% TTO lotion, placebo lotion and 100% macadamia oil. All subjects were tested for approximately 8 nickel reaction sites and 2 or 3 sites without nickel over an area of 300 cm². There were always at least two of the 8 nickel reactions sites to which nickel only was applied and which served as control sites. Sites without nickel were single tests and served as control sites for the TTO, 5% TTO lotion, placebo lotion (no TTO) and macadamia oil. All reaction sites to nickel that were treated with one of the above, were tested in duplicate.

Inflammatory reactions to nickel were measured on days 3, 5 and 7 after nickel sulphate application (same time of day on all occasions). The size of the reactions (longitudinal and horizontal diameters) was measured with calipers (Mitutoyo Corp., Tokyo, Japan) and the flare area was calculated using the formula¹⁷:

$$\text{Flare area (cm}^2\text{)} = \pi/4 \times [(D_1+D_2)/2]^2$$

where D_1 and D_2 represent the longitudinal and horizontal diameters of the flare (cm), respectively.

The intensity of the erythema was measured using a fibre optic tissue spectrum analyzer (Sumitomo Electric, Japan) which measured the concentration of haemoglobin in skin. The measuring probe consisted of a fibre-optic cable (2 mm in diameter) mounted in a plastic disk which measured 1 cm in diameter. Measurements for each reaction site were made for 5 seconds (3 readings/second) and in duplicate. The erythema index was determined by subtracting the mean reading of normal skin (adjacent to the reaction site) from the mean reading of the reaction site. After the first and second reading (days 3 and 5 respectively), 25 μL of 100% TTO, 100% macadamia oil, 5% TTO lotion, or placebo lotion were dropped onto the assigned reaction sites and left to dry (30 min).

Identification of cellular influx

In one nickel-sensitive individual, two inflamed sites on the buttock induced by nickel application were biopsied (4 mm) on day 4. One nickel-treated site was used as a control, the other site was treated with 100% TTO on day 3. EI measurements were taken on days 3 and 4.

The 4 mm skin biopsies were fixed in 10% buffered formalin, paraffin embedded with a vertical orientation, and 4 μm consecutive sections were stained immunohistochemically. Sections were treated with murine anti-CD-4 or -CD-8 antibodies (Novocastra Laboratories, Newcastle upon Tyne, UK), followed by biotinylated goat anti-mouse antibodies and the Ultra Streptavidine Detection System (Signet Pathology Systems Inc, Dedham, MA). Control sections were stained in parallel without primary antibodies.

Assay of proliferative response to nickel sulfate by PBMC

Mononuclear cells were isolated from heparinised venous blood by density gradient centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway).²¹ PBMC (2×10^5 cells in 140 μL) were suspended in RPMI-1640 medium (Cytosystems, Castle Hill, Australia) supplemented with 13.3 mM NaHCO_3 , 2 mM glutamine, 50 μM β -mercaptoethanol, 100 U mL^{-1} penicillin, 100 $\mu\text{g mL}^{-1}$ streptomycin, and 2 nM 3-(*N*-morpholino)propanesulphonic acid with an osmolality of 290 mmol kg^{-1} H_2O ('complete RPMI'), and 10% heat-inactivated fetal calf serum (56°C for 30 min), were added to wells of a 96-well round-bottom plate (Nunclon, Nunc A/S, Roskilde, Denmark). Nickel sulphate (Sigma Chemical Co, St Louis, MO) in 50 μL complete RPMI was added to a final concentration of 6.25 or 1.25 $\mu\text{g mL}^{-1}$. Polyclonal mitogens were diluted in complete RPMI and added in 50 μL to reach the following final concentrations: 15 $\mu\text{g mL}^{-1}$ phytohaemagglutinin (PHA), 75 $\mu\text{g mL}^{-1}$ concanavalin A (conA), and 2.5 $\mu\text{g mL}^{-1}$ pokeweed mitogen (PWM) (Sigma). For all experiments PBMC from at least one nickel-sensitive subject and one control subject were run in parallel. The cultures with nickel or polyclonal mitogens were incubated at 37°C in 5% CO_2 and harvested after 4 days. ^3H -thymidine (1 μCi in 10 μL) (specific activity 2.0 Ci mmol^{-1}) diluted in complete RPMI was added to each well 24 hours prior to harvesting on a microplate harvester (Tomtec, Orange, CT) using glass fibre filters. Filters were dried and assayed in a scintillation counter (Wallac 1205 betaplate, Wallac Oy, Turku, Finland). The mononuclear cell response was calculated as the mean of counts per min (cpm) of quadruplicate cultures. Day 4 was chosen for harvesting because at day 3, levels of ^3H -thymidine incorporation were very low. By day 7, levels of ^3H -thymidine incorporation were very high and good discrimination between control wells and treatment wells had been lost.

Assay of the effect of the water soluble components of TTO

Wells of 2×10^5 cells in 190 μL (as described above) were incubated with 20 μL of the water-soluble components of TTO (1.25% v/v). For direct comparison, identical wells were incubated with 20 μL complete RPMI.

Measurement of toxicity of TTO

Metabolically active cells were enumerated using the CellTiter 96 AQueous non-radioactive cell proliferation assay (Promega Corporation, Madison, WI). Mononuclear cells ($2 \times 10^5/210 \mu\text{L}$) were cultured for 4 days with nickel sulfate ($6.25 \mu\text{g mL}^{-1}$), with or without TTO (0.125%). The MTS/PMS substrate (20 μL) was added and the amount of formazan product measured spectrophotometrically at 490 nm after 1.5 h, 2.5 h and 3 h. Additionally, mononuclear cells ($2 \times 10^5/210 \mu\text{L}$) cultured for 4 days were examined microscopically after addition of trypan blue (0.05%) (Sigma). Cells that stained with trypan blue were considered to be non-viable and representative of the degree of cell death.

Statistical analyses

For comparison of responses at different treatment sites of a subject's back, the Student's paired *t*-test was used. For comparison of responses by PBMC to different antigen/mitogens, the Student's paired *t*-test was used. A value of $P < 0.05$ was considered significant.

3.2 Results

Intra-individual variability in a CHS response to nickel sulphate and TTO

There was both inter- and intra-individual variation in response to nickel for a CHS response. The results presented in Table 2 indicate inter-individual variation in CHS response to nickel and any regulatory effect of TTO. An example of intra-individual variation is shown in Table 3, where the FA and EI for 3 sites treated with nickel-only at different positions on the back were compared.

Table 2. Mean EI and FA (cm²) of the nickel-induced CHS reactions for 18 subjects treated with 25 µL of 100% TTO on days 3 and 5 after nickel contact.

SUBJECT ^a	TIME	Erythema index (EI)			Flare Area (FA)			CONC OF Ni USED ^b
		NICKEL ONLY	NICKEL + TTO	NICKEL + MAC	NICKEL ONLY	NICKEL + TTO	NICKEL + MAC	
1	day 3	13.04	21.37		1.27	2.45		5%
	day 5	13.08	8.48		0.88	1.57		
	day 7	10.24	9.45		0.88	1.01		
3	day 3	17.70	15.02		2.26	2.65		0.50%
	day 5	16.78	11.52		2.08	1.90		
	day 7	10.38	5.77		1.42	1.42		
4	day 3	18.93	16.31		2.37	1.65		0.125%
	day 5	23.39	27.07		2.17	1.68		
	day 7	15.21	14.54		1.77	1.74		
6	day 3	15.98	17.33		1.29	1.13		5%
	day 5	18.21	22.56		1.46	1.42		
	day 7	12.71	13.77		1.42	0.88		
7	day 3	18.20	17.42		1.66	1.34		0.125%
	day 5	21.39	15.96		1.14	0.72		
	day 7	12.46	10.91		1.01	0.55		
8	day 3	19.30	11.42		0.37	0.77		5%
	day 5	18.30	19.64		0.77	0.88		
	day 7	15.80	12.64		0.41	0.77		
10	day 3	21.33	21.41		1.01	0.79		0.50%
	day 5	20.74	22.59		1.01	0.72		
	day 7	10.37	13.36		0.89	0.89		

(Table continued next page)

^a Subjects grouped according to whether they exhibited a prolonged development phase of CHS defined by an EI at day 7 greater than 100% of day 3 for the nickel-only sites.

^b concentration of nickel sulphate required to induce a moderate confluent erythematous CHS response.

Table 2 (continued). Mean EI and FA (cm²) of the nickel-induced CHS reactions for 18 subjects treated with 25 µL of TTO (100%) on days 3 and 5 after nickel contact.

SUBJECT ^a	TIME	Erythema index (EI)			Flare Area (FA)			CONC OF Ni USED ^b
		NICKEL ONLY	NICKEL + TTO	NICKEL + MAC	NICKEL ONLY	NICKEL + TTO	NICKEL + MAC	
11	day 3	24.33	11.17	13.09	0.97	0.48	0.17	0.06%
	day 5	12.27	7.70	9.01	0.97	0.36	0.22	
	day 7	13.03	11.85	11.41	0.95	0.36	0.22	
12	day 3	20.63	18.05	20.82	2.81	2.76	2.86	1%
	day 5	15.57	9.92	13.38	1.68	1.83	1.61	
	day 7	10.50	7.36	10.86	0.96	1.01	0.81	
13	day 3	11.05	8.71	13.43	2.09	1.58	2.45	0.50%
	day 5	8.57	5.03	7.46	1.79	1.04	1.59	
	day 7	8.12	5.35	6.19	1.73	0.76	1.29	
16	day 3	18.60	17.47		3.24	2.53		2%
	day 5	18.38	17.23		3.56	1.69		
	day 7	8.28	6.40		2.07	1.38		
18	day 3	24.01	21.70	23.70	2.48	1.74	1.82	5%
	day 5	22.30	22.30	20.70	2.40	1.50	2.08	
	day 7	20.15	13.85	15.72	1.68	0.85	1.29	

(Table continued next page)

^a Subjects grouped according to whether they exhibited a prolonged development phase of CHS defined by an EI at day 7 greater than 100% of day 3 for the nickel-only sites.

^b concentration of nickel sulphate required to induce a moderate confluent erythematous CHS response.

Table 2 (continued). Mean EI and FA (cm²) of the nickel-induced CHS reactions for 18 subjects treated with 25 µL of TTO (100%) on days 3 and 5 after nickel contact.

SUBJECT ^a	TIME	Erythema index (EI)			Flare Area (FA)			CONC OF NI USED ^b
		NICKEL ONLY	NICKEL + TTO	NICKEL + MAC	NICKEL ONLY	NICKEL + TTO	NICKEL + MAC	
SUBJECTS WITH A PROLONGED DEVELOPMENT PHASE OF CHS								
2	day 3	27.4	23.77		3.77	2.45		0.25%
	day 5	37.14	16.74		3.78	2.86		
	day 7	30.7	7.99		1.01	0.77		
5	day 3	6.49	0		1.01	0.19		0.125%
	day 5	17.06	17.31		0.88	0.25		
	day 7	17.41	1.35		1.13	0.32		
9	day 3	8.40	24.6		1.42	4.28		0.50%
	day 5	25.74	19.07		1.13	1.90		
	day 7	18.99	15.38		0.88	0.66		
14	day 3	19.69	20.39	21.71	2.52	2.52	2.46	0.25%
	day 5	23.10	18.14	26.46	2.40	1.50	1.91	
	day 7	22.26	15.89	26.17	2.09	1.50	1.91	
15	day 3	16.28	18.13	19.34	5.10	6.60	4.34	1%
	day 5	22.01	18.64	31.43	2.28	1.50	3.19	
	day 7	17.33	13.99	23.34	2.36	1.59	3.02	
17	day 3	13.82	13.29	17.52	2.48	2.93	2.09	5%
	day 5	17.00	11.66	21.94	2.37	1.20	2.04	
	day 7	26.34	15.39	21.14	1.52	1.01	1.02	

(End of Table 2)

^a Subjects grouped according to whether they exhibited a prolonged development phase of CHS defined by an EI at day 7 greater than 100% of day 3 for the nickel-only sites.

^b concentration of nickel sulphate required to induce a moderate confluent erythematous CHS response.

Table 3 Intra-individual variation in mean EI and FA of nickel-induced CHS reactions measured on days 3, 5 and 7.

SITE	EI			FA (cm ²)		
	day 3	day 5	day 7	day 3	day 5	day 7
1*	23.95	16.92	10.91	2.65	1.57	0.88
2*	14.89	17.00	10.59	2.26	1.57	0.88
3*	23.06	12.78	9.99	3.53	1.90	1.13
Mean	20.63	15.57	10.50	2.81	1.68	0.96
SD	4.99	2.41	0.47	0.65	0.19	0.14
Coeff. of Variation	24%	16%	5%	23%	11%	15%

* The three indicated sites represent different locations on the back of a nickel-sensitized individual challenged with 1% NiSO₄.

Effect of 100% TTO on nickel-induced EI

The mean EI of the TTO-treated sites for the 18 nickel-sensitive subjects listed in Table 2 was significantly less than that for the nickel only test sites at both day 5 and day 7 ($P=0.02$ and $P=0.005$ respectively) (Fig. 1A). In 6 subjects the response to nickel after 7 days was greater than at day 3. The EI of nickel responses, with or without TTO application, of these subjects who exhibited a prolonged development phase of their CHS response to nickel is shown in Fig. 1B. The difference in EI at day 5 was close to significance ($P=0.055$), and at day 7 was significant ($P=0.02$). The effect of TTO treatment on the nickel reactions was to reduce the EI at day 7 to 50% of the EI of the nickel only reactions (and to approximately 70% for all 18 subjects in Fig 1A). When the effect of TTO on erythema of the remaining 12 patients were examined, there was no difference between the TTO-treated sites and the nickel only sites (Fig. 1C). An analysis of the degree of nickel sensitivity of the groups presented in Figs. 1B and 1C, as determined by the concentration of nickel sulphate required to elicit a CHS response, indicated that a similar range of nickel concentration was required in each group (Table 2).

Macadamia oil (100%) was tested as a control for TTO (100%). For 7 of 7 subjects, the mean EI was not significantly different between the nickel-treated sites and those treated with both nickel and macadamia oil (Fig. 1D). Three of the 7 subjects treated with macadamia oil exhibited a prolonged development phase of CHS. There was no difference observed in the response to macadamia oil treatment by these subjects.

For all 10 nickel-sensitive subjects investigated with a diluted TTO preparation, administration of a 5% TTO lotion or a placebo lotion was without effect on the EI of a nickel CHS response (data not shown).

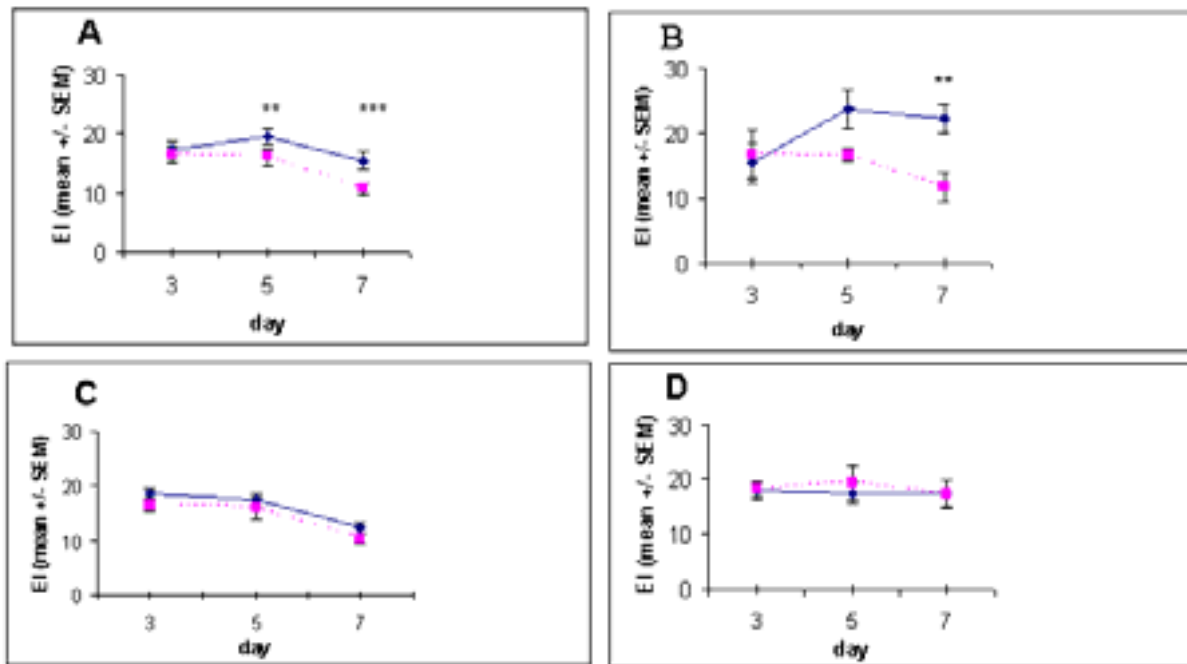


Figure 1. The effect of topical administration of 100% TTO (25 μ L) on the erythematous response of a nickel-induced CHS in nickel-sensitive subjects measured on days 3, 5 and 7 after nickel contact (n=18). The EI are shown as mean \pm SEM. In **A**, the mean EI of the TTO-treated nickel site (broken line) was significantly less than the nickel-only site (solid line) at both days 5 and 7. In **B**, the response to TTO treatment for the 6 subjects exhibiting a prolonged development phase of CHS, was significantly less for the TTO-treated site (broken line) compared to the nickel-only site (solid line). In **C**, there was no significant response for the remaining 12 subjects treated with TTO (broken line) compared to the nickel-only site (solid line). The data for treatment with 100% macadamia oil (broken line) are shown in **D** (n=7). The significance of the difference in means on any day is indicated by asterisks: (**) $P < 0.02$; (***) $P < 0.01$.

Effect of 100% TTO on nickel-induced FA

For all 18 subjects studied, there was a significant difference at both days 5 and 7 between the mean FA of the TTO-treated nickel sites and that of the nickel only sites ($P=0.008$ and $P=0.0008$, respectively) (Fig. 2A). When the FA for the 6 patients who exhibited a prolonged phase of CHS development was studied, a significant reduction in mean FA was noted for the TTO-treated nickel sites at day 7 ($P=0.003$) (Fig. 2B). Significance was also seen for the effect of TTO on the mean FA response to nickel for the other 12 subjects at day 7 ($P=0.04$) (Fig. 2C).

As for the EI, there was no significant difference in mean FA between the nickel-treated sites and those treated with both nickel and macadamia oil (Fig. 2D). There was also no significant difference in mean FA between sites treated with nickel and a placebo lotion, and those treated with nickel and a 5% TTO lotion (data not shown).

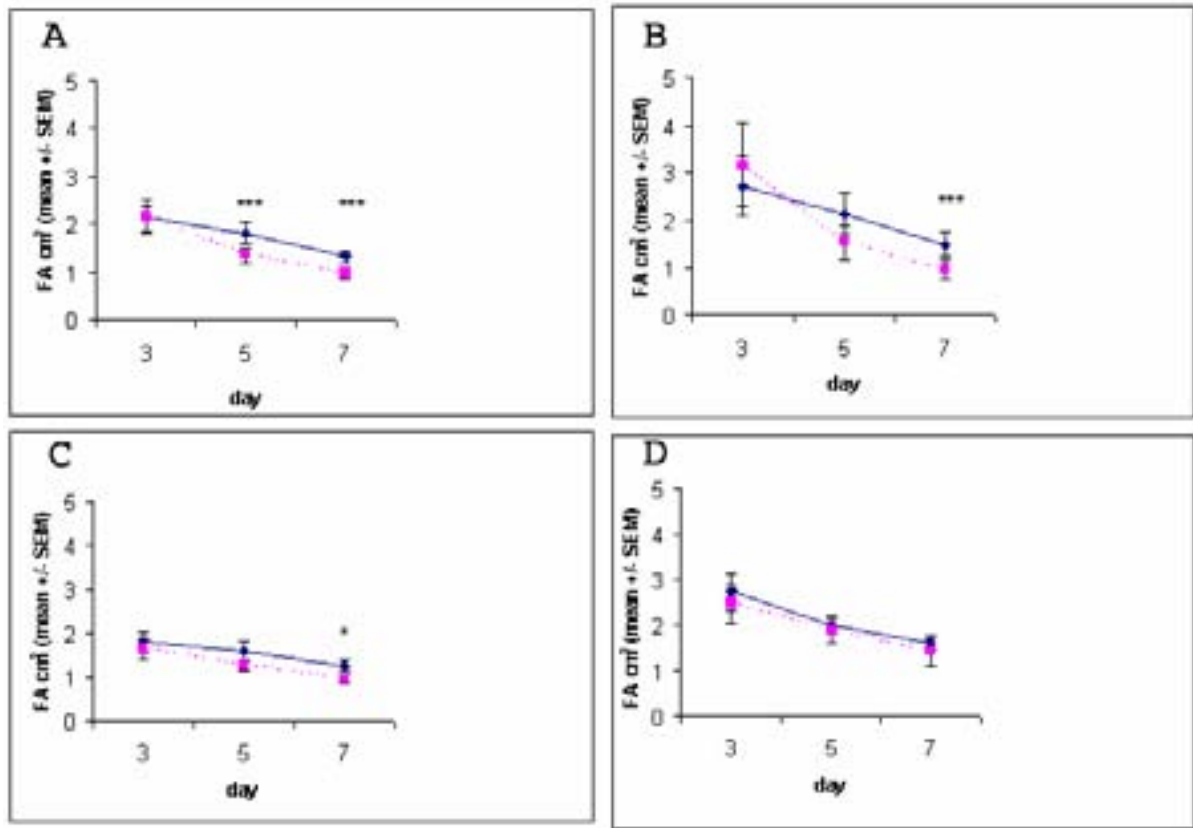


Figure 2. The effect of topical administration of 100% TTO (25 μ L) on the mean flare area (FA) of the nickel-induced CHS in nickel-sensitive subjects on days 3, 5 and 7 after nickel contact. The FA is represented as the mean \pm SEM (cm^2). In **A**, in which data from all 18 subjects are presented, the TTO-treated nickel site (broken line) was significantly less than the nickel-only site (solid line) at both days 5 and 7. In **B**, as for the EI, the response to TTO treatment for the 6 subjects exhibiting a prolonged development phase of CHS, was significantly less for the TTO-treated site (broken line) compared to the nickel-only site (solid line). The response for the remaining 12 subjects, shown in **C**, illustrates a smaller, but still significant reduction in the TTO-treated sites at day 7. The data for treatment with 100% macadamia oil are shown in **D** (n=7). The significance of the difference in means on any day is indicated by asterisks: (*) $P < 0.05$; (***) $P < 0.01$.

Effect of TTO on nickel-induced lymphocyte influx

The effect of TTO administration on lymphocyte influx into a CHS site was examined in a single subject. On day 4 after nickel administration, there was a significant influx of both CD4-positive and CD8-positive cells. Administration of 100% TTO on day 3 significantly reduced the prevalence of CD4-positive cells (Fig. 3).

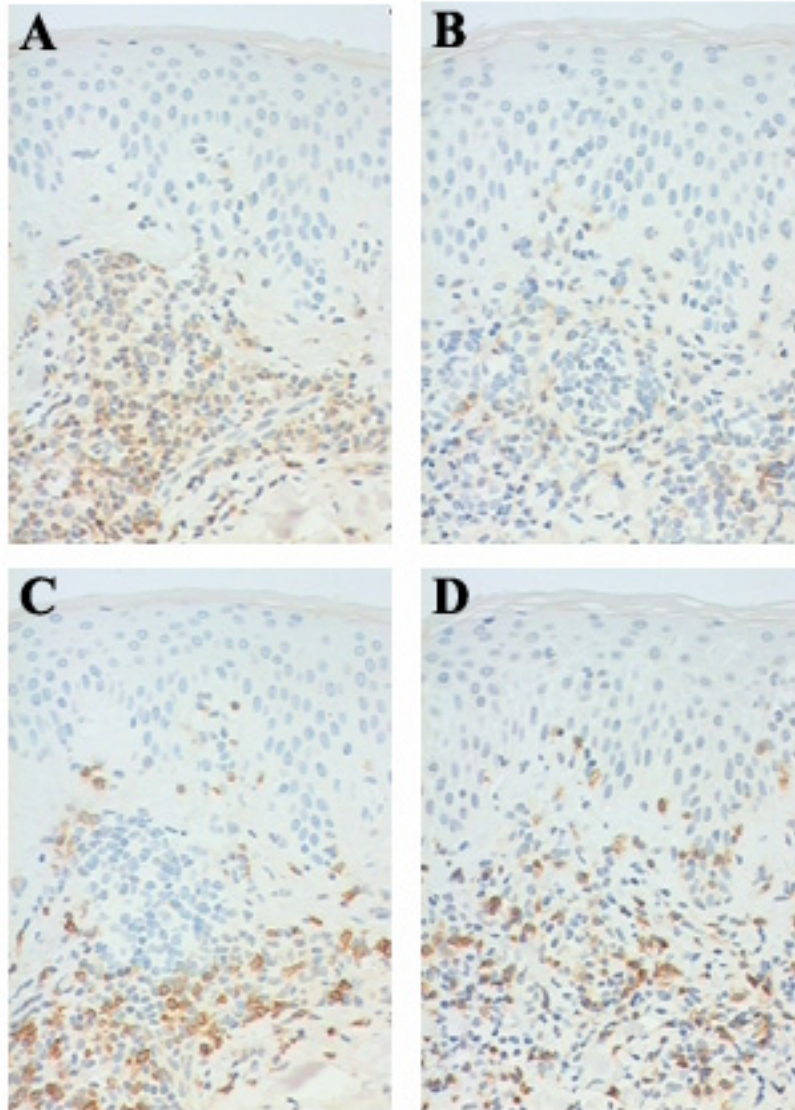


Figure 3. The effect of topical administration of 100% TTO on cellular influx in nickel-induced CHS. Consecutive histological sections (4 μ m) were immunohistochemically stained for CD4⁺ (top row) and CD8⁺ (bottom row) T-lymphocytes. **A and C** represent consecutive sections for the nickel-only site. **B and D** represent consecutive sections for the TTO-treated site. There is a marked reduction in the CD4⁺ T-lymphocyte infiltrate. The EI measured on days 3 (1 h post removal of nickel patches) and 4 (pre-biopsy) for each site is shown in Table 4.

Table 4 The mean EI for nickel-induced CHS reactions on the buttock of a single subject.

	Nickel-only	Nickel+100% TTO
Day 3 ^a	31.1	36.1
Day 4 ^b	27.6	25.0

^a 1 h post removal of nickel patches

^b pre-biopsy

Effect of TTO on nickel-induced proliferation by mononuclear cells

There was no significant proliferation above background by PBMC from non-nickel-sensitive subjects ($n=6$) to either concentration of nickel sulphate (1.25 and $6.25 \mu\text{g mL}^{-1}$) (Fig. 4A). In the nickel-sensitive group ($n=11$) there was significant proliferation above background by mononuclear cells incubated with $6.25 \mu\text{g mL}^{-1}$ nickel ($p=0.002$) and with $6.25 \mu\text{g mL}^{-1}$ nickel + TTO (0.125%) ($p=0.01$, Fig. 4B). Nickel sulphate at the lower concentration ($1.25 \mu\text{g mL}^{-1}$) did not induce significant proliferation (Fig. 4 B).

The proliferative response of PBMC to $6.25 \mu\text{g mL}^{-1}$ nickel for the nickel-sensitive subjects was significantly reduced by addition of TTO (0.125% (v/v)) ($P=0.013$). However, the PBMC proliferative response remained significantly higher than for the TTO control group ($P=0.009$). There was no significant effect of TTO on PBMC proliferation to nickel at $1.25 \mu\text{g mL}^{-1}$.

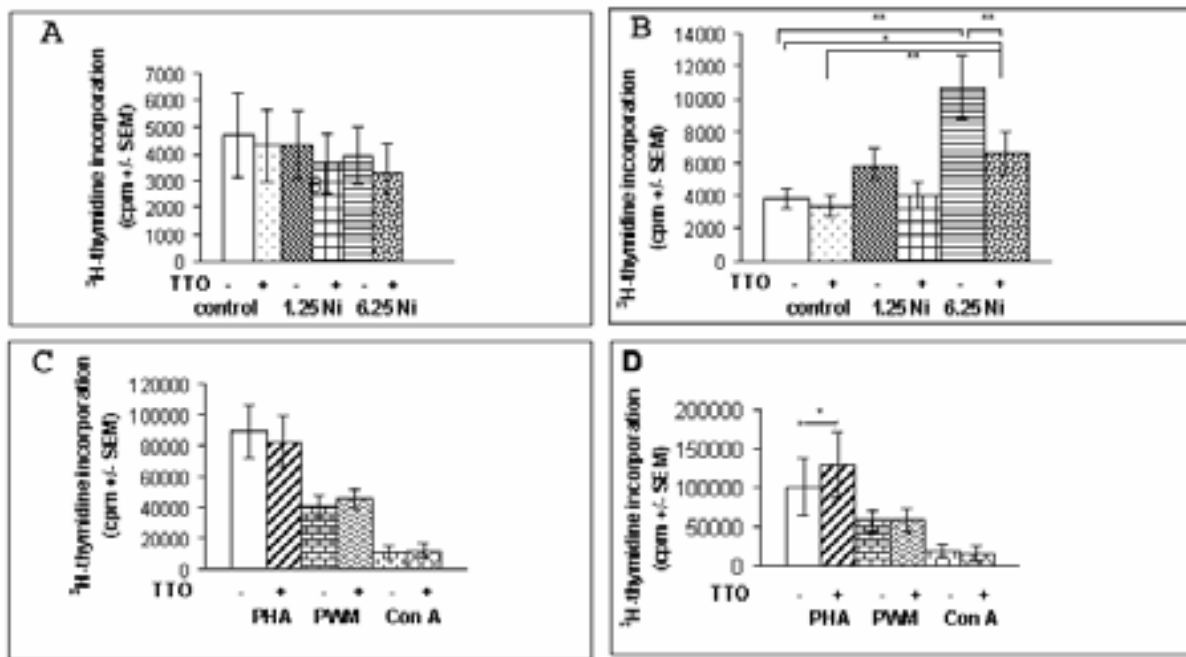


Figure 4. The effect of TTO on nickel-induced ^3H -thymidine incorporation by PBMC from both nickel-sensitive ($n=11$) and non-nickel-sensitive subjects ($n=6$). PBMC ($2 \times 10^5/200 \mu\text{L}$) were cultured for 4 days with nickel (1.25 and $6.25 \mu\text{g mL}^{-1}$), and polyclonal mitogens (PHA, PWM, and Con A), with or without TTO (0.125%). In **A**, for non-nickel-sensitive subjects, there was no significant proliferation of PBMC to either concentration of nickel. In **B**, for nickel-sensitive subjects, PBMC proliferated significantly in response to nickel ($6.25 \mu\text{g mL}^{-1}$). Treatment with TTO significantly reduced the PBMC proliferation to $6.25 \mu\text{g mL}^{-1}$ nickel. However, the PBMC proliferation for $6.25 \mu\text{g mL}^{-1}$ nickel + TTO remained significantly higher than the control. **C** and **D** shows the PBMC proliferation to polyclonal mitogens for the non-nickel-sensitive subjects ($n=6$), and the nickel-sensitive subjects ($n=11$) respectively with or without TTO. (* $) P = 0.02$; (** $) P < 0.01$

The TTO effect was not due to toxicity because no cell death was seen when cell cultures were analysed by trypan blue exclusion immediately before harvesting on day 4. Additionally, MTS assays performed on day 4, showed no change in metabolic activity of the cells treated with TTO (Fig. 5).

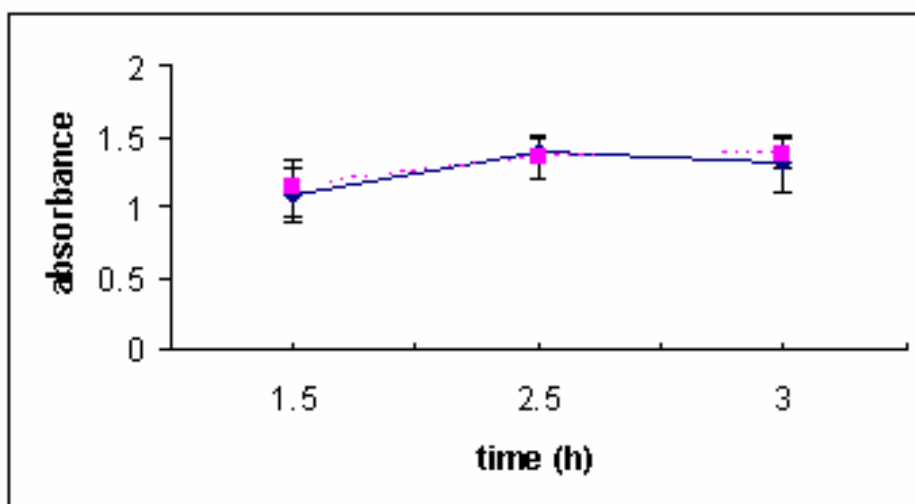


Figure 5. The effect of TTO on metabolic activity of PBMC. PBMC ($2 \times 10^5/200 \mu\text{L}$) were cultured for 4 days with nickel ($6.25 \mu\text{g mL}^{-1}$) with or without TTO (0.125%). Metabolically active cells were enumerated using the CellTiter 96 AQueous non-radioactive cell proliferation assay. The amount of formazan product was measured spectrophotometrically at 490 nm after 1.5 h, 2.5 h and 3 h. There was no difference in the metabolic activity of cells incubated with TTO (broken line) compared to those without TTO (solid line).

Effect of TTO on mitogen-induced proliferation by PBMC

There was significant proliferation in response to all polyclonal mitogens by PBMC from both the nickel-sensitive and non-nickel-sensitive subjects (Fig. 4 C,D). No significant difference in PBMC proliferation to polyclonal mitogens was seen between the nickel-sensitive and control groups. Treatment with TTO (0.125%) significantly increased the PBMC proliferation to PHA in the nickel-sensitive group ($P=0.01$) but otherwise was without significant effect and in no instance was an inhibitory effect of TTO observed.

3.3 Discussion

In this study, topical 100% TTO had significant anti-inflammatory effects on nickel-induced CHS. The anti-inflammatory effect was most evident in a subgroup (30%) of nickel-sensitive individuals who exhibited a prolonged development phase of CHS defined by a larger EI of the nickel-treated sites at day 7 compared to day 3. We hypothesise that sustained antigen presentation to nickel-specific T-lymphocytes by cells in the skin such as macrophages, Langerhans cells and keratinocytes drives the prolonged development of CHS in these individuals. It is possible that TTO may affect the antigen presenting cells or the antigen presenting process. The finding that TTO (0.125%) significantly reduced the proliferation of PBMC to $6.25 \mu\text{g mL}^{-1}$ nickel but not to mitogens for all nickel-sensitive volunteers supports this theory. For the 10 subjects who participated in both studies, there was no correlation between severity of nickel-sensitivity and the level of nickel-induced mononuclear cell proliferation, or the regulatory effect of TTO (data not shown).

When considering the regulatory effect of TTO on CHS development or resolution, there are multiple effectors that culminate in whole body responses. Integration of the vascular and neuronal systems in addition to the immune system are responsible for full CHS development, and TTO may be affecting any or all of these elements. In the present study, the investigations of the regulatory effect of TTO on the proliferation of PBMC, explored only one part of this integrated system. Previous work carried out in this laboratory has shown that TTO can regulate the oedema associated with the efferent phase of a CHS in mice.¹³ In mice sensitized to the chemical hapten, trinitrochlorobenzene (TNCB), a single topical application of 100% TTO to the TNCB challenge site significantly reduced the development of skin oedema.¹³ Undiluted TTO was found to be effective in reducing the CHS-associated swelling

when added 30 min before, and up to 7 h after challenge with TNCB. Additional murine studies have shown TTO suppressed histamine-induced oedema in the ears of mice, and this inhibitory effect was sustained in capsaicin-treated (neuropeptide-depleted) mice.¹² Therefore, in mice, the anti-oedema effect of TTO was not due to regulation of the cutaneous sensory nerves. In the present study, application of TTO (at day 3 and day 5) occurred much later than in the murine studies. Whether earlier application of 100% TTO to the CHS reaction sites would result in greater anti-inflammatory effects, or therapeutic benefit in more subjects, remains to be elucidated.

Due to their small size, metal ions are incomplete antigens (haptens) which have to bind to endogenous peptides to become full antigens.¹⁴ They associate in a trimolecular complex of MHC II molecules, peptide, and T cell receptor to induce a specific T cell response. It has recently been reported that the development of a delayed contact dermatitis to haptens depends upon the balance between CD8⁺ T lymphocytes with cytotoxic activity and CD4⁺ T cells, which comprise both effector and regulatory cells.²² Following antigen presentation by nickel-exposed MHC class II-bearing APC, naïve CD4 cells are converted to antigen specific Thelper 1 (Th1) effector cells following the action of IFN γ and perhaps IL-12.²³ TTO may suppress the production of chemokines and cytokines such as TNF α , IL-12 or IFN γ which may account for the profound reduction in CD4⁺ T lymphocytes in the biopsy from TTO-treated skin; TTO has been previously reported to suppress TNF α production by human monocytes.⁹

Investigations into nickel-specific T cell clones derived from blood or skin showed that circulating and skin-infiltrating nickel-specific T cells have a nonrandom pattern of recirculation and modulate their cytokine pattern after emigration from the peripheral blood into the skin.²⁴ The cytokine production (Th1 vs Th2) depends upon the microenvironment of the lymphocytes and plays a critical role in directing T cell responses.¹⁴ Although the murine studies did not show TTO to affect the cellular infiltrate of a CHS response to TNCB, in the present human study, treatment with TTO clearly resulted in a reduction of the CD4⁺ infiltrate into the nickel-inflamed skin. This study suggested that TTO affects the balance of infiltrating T lymphocytes which in turn alters the balance of cytokine production and inflammatory response to nickel.

No adverse skin reactions to TTO were reported during the study. However, a small proportion of the human population (<5%) will experience adverse skin reactions (irritant or allergic contact dermatitis) following topical application of undiluted, and to a lesser extent 5% or 10% TTO.²⁵⁻²⁶ Many of these adverse reactions are associated with exposure to the oxidized degradation products of TTO.²⁵⁻²⁶ Of the 18 subjects in this study, 44% had used TTO-containing products prior to the study and no-one reported previous reactions to TTO. In this study, the risk of oxidation of TTO was reduced by storage in 25 ml brown bottles kept at 4°C, protected from UV radiation, and discarded after one week of use.

Of the subjects who had used TTO previously, 75% reported TTO to be effective in reducing itch. Attempts made to quantify the effect of TTO on itch in the present study were limited by poor localization of itch sensation on the skin of the back.

No oil could be considered the ideal control oil for TTO. In a previous study liquid paraffin oil (B.P) was used as it was considered the best example of an immunologically inert oil (Chapter 2). In an attempt to better match the volatility of TTO, 100% triple filtered refined macadamia oil was used as it is less occlusive than paraffin oil. As with the paraffin oil, no therapeutic benefit from treatment with macadamia oil (100%) was shown.

To our knowledge this is the first human study to show that 100% TTO has anti-inflammatory effects in CHS of the skin due to exposure to a chemical hapten in sensitized individuals. The anti-bacterial and anti-fungal properties of TTO are already well established.²⁻⁴ The finding that TTO has anti-inflammatory in addition to anti-microbial effects, maximizes its therapeutic benefit for conditions such as nickel-induced contact dermatitis.

Further studies are required to investigate if application of TTO earlier in the course of development of a CHS response would elicit a significant reduction in CHS inflammation in more individuals. Also, further investigations focusing on the anti-inflammatory properties of the individual water-soluble components of TTO are indicated. We hypothesise that topical application of undiluted TTO immediately after known contact with nickel containing items may be beneficial in reducing the CHS response in nickel-sensitive individuals. Additionally, other contact dermatitis conditions to specific allergens, such as those secondary to cobalt, chromate, rubber, resins, cosmetics, plants and woods may be treated by topical TTO with therapeutic benefit.

Chapter 4: References

1. Carson CF, Riley TV. The antimicrobial activity of tea tree oil. *Med J Aust* 1994; **160**: 236.
2. Carson CF, Cookson BD, Farrelly HD, Riley TV. Susceptibility of methicillin-resistant *Staphylococcus aureus* to the essential oil of *Melaleuca alternifolia*. *J Antimicrob Chemother* 1995; **35**: 421-424.
3. Hammer KA, Carson CF, Riley TV. Susceptibility of transient and commensal skin flora to the essential oil of *Melaleuca alternifolia*. *Amer J Infect Control* 1996; **24**: 186-189.
4. Concha JM, Moore LS, Holloway WJ. Antifungal activity of *Melaleuca alternifolia* (tea tree) oil against various pathogenic organisms *Podiatr Med Assoc* 1998; **88**: 489-492.
5. Ernst E, Huntley A. Tea tree oil: A systematic review of randomized clinical trials. *Forsch Komplementarmed* 2000; **7**: 17-20.
6. Bassett IB, Pannowitz DL, Barnetson RStC. A comparative study of tea tree oil versus benzoylperoxide in the treatment of acne. *Med J Aust* 1990; **153**: 455-458.
7. Tong MM, Altman PM, Barnetson RStC. Tea tree oil in the treatment of tinea pedis. *Australas J Dermatol* 1992; **33**: 145-149.
8. Nenoff P, Hausteil UF, Brandt W: Antifungal activity of the essential oil of *Melaleuca alternifolia* (tea tree oil) against pathogenic fungi in vitro. *Skin Pharmacol* 1996; **9**: 366-394.
9. Hart PH, Brand C, Carson CF, Riley TV, Prager RH, Finlay-Jones JJ. Terpinen-4-ol, the main component of the essential oil of *Melaleuca alternifolia* (tea tree oil), suppresses inflammatory mediator production by activated human monocytes. *Inflamm Res* 2000; **49**: 619-26.
10. Brand C, Ferrante A, Prager RH, Riley TV, Carson CF, Finlay-Jones JJ, Hart PH. The water-soluble components of the essential oil of *Melaleuca alternifolia* (tea tree oil) suppress the production of superoxide by human monocytes, but not neutrophils, activated in vitro. *Inflamm Res* 2001; **50**: 213-9.
11. Clough GF, Bennett AR, Church MK. Effects of H₁ antagonists on the cutaneous vascular response to histamine and bradykinin: a study using scanning laser Doppler imaging. *Br J Dermatol* 1998; **138**: 806-14.
12. Brand C, Townley SL, Finlay-Jones JJ, Hart PH. Tea tree oil reduces histamine-induced oedema in murine ears. *Inflamm Res* 2002; **51**: 283-9.
13. Brand C, Grimbaldston MA, Gamble JR, et al. Tea tree oil reduces the swelling associated with the efferent phase of a contact hypersensitivity response. *Inflamm Res* 2002; **51**: 236-44.
14. Bùdinger L, Hertl M. Immunologic mechanisms in hypersensitivity reactions to metal ions: an overview. *Allergy* 2000; **55**: 108-15.
15. Simonetti V, Manzini BM, Seidenari S. Patch testing with nickel sulfate: comparison between 2 nickel sulfate preparations and 2 different test sites on the back. *Contact Dermatitis* 1998; **39**: 187-91.
16. Heffler LC, Kastman AL, Jacobsson Ekman G, et al. Langerhans cells that express matrix metalloproteinase 9 increase in human dermis during sensitization to diphenylcyclopropanone in patients with alopecia areata. *Br J Dermatol* 2002; **147**: 222-9.
17. Marshman G, Burton JL, Archer CB. Comparison of the actions of kallidin and bradykinin in the skin of normal and psoriatic subjects. *Clin Exp Dermatol* 1996; **21**: 112-5.
18. Elliott MJ, Maini RN, Feldmann M *et al.*: Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor . *Arth Rheum* 1993; **12**: 1681-90.
19. Downs AMR, Stafford KA, Coles GC: Monoterpenoides and tetralin as pediculocides. *Br J Dermatol* 1999; **141**(suppl 55): 104.
20. Essential Oils – *Oil of Melaleuca*, terpinen-4-ol (tea tree oil). ISO-4370, 1996. International Organisation for Standardisation, Geneva, Switzerland.
21. Boyum A. Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* 1968; **97**: 77-80.

22. Sebastiani S, Alvanesi C, Nasorri F, et al. Nickel specific CD4+ and CD8+ T cells display distinct migratory responses to chemokines produced during allergic contact dermatitis. *J Invest Dermatol* 2002; 118: 1952-58.
23. Sosroseno W. The immunology of nickel-induced allergic contact dermatitis. *Asian Pac J Allergy Immunol* 1995; 13: 173-81.
24. Werfel T, Huntschel M, Renz H, Kapp A. Analysis of phenotype and cytokine pattern of blood- and skin-derived nickel specific T cells in allergic contact dermatitis. *Int Arch Allergy Immunol* 1997; 113: 384-86.
25. Southwell IA, Freeman S, Rubel D. Skin irritancy of tea tree oil. *J Essent Oil Res* 1997; 9: 47-52.
26. Hausen BM, Reichling J, Harkenthal M. Degradation products of monoterpenes are the sensitizing agents in tea tree oil. *Am J Contact Dermatitis* 1999; 10: 68-77.