RURAL INDUSTRIES RESEARCH
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The anti-inflammatory and anti-itch properties of tea tree oil

- in vivo studies

A report for the Rural Industries Research and Development Corporation

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Foreword

Laboratory and clinical studies have in recent times confirmed the anecdotal anti-microbial properties of tea tree oil. Anecdotal evidence has also 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 activities of human white blood cells. As these cells can participate in inflammatory responses in skin, the potential for beneficial outcomes with tea tree oil treatment seemed promising.

In the present report, the focus moves from laboratory-based experiments to studies of inflammatory responses in an animal model. Tea tree oil was found to limit 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 initiation of the inflammation, which would fit the likely clinical use. It is also important to note that tea tree oil did not lessen the swelling associated with sunburn.

In summary, this report provides further evidence that tea tree oil has potential as an anti-inflammatory agent. With the results of previous studies, it is an excellent foundation for further work in humans.

This project was funded from industry revenue that is matched by funds provided by the Federal Government.

This report, a new addition to RIRDC's diverse range of over 800 research publications, forms part of our Tea Tree Oil R&D program

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Peter Core
Managing Director
Rural Industries Research and Development Corporation

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Abbreviations

CHS	contact hypersensitivity
H&E	haematoxylin and eosin
SCFT	subcutaneous fatty tissue
TNCB	trinitrochlorobenzene
TTO	tea tree oil
TNFα	tumour necrosis factor α
UVB	ultraviolet B

Contents

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 [1-3], with now many publications detailing the susceptibility of various microbes to tea tree oil.

Anecdotal evidence has also 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.

Earlier studies, also supported by RIRDC, focussed on human white blood cells, which 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 activation of intracellular mechanisms that kill germs. 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 (it would not impair the ability of neutrophils to kill germs). They also indicated a potential role as an anti-inflammatory agent, by regulating 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 [6]. These observations plus the results of the experiments on human white blood cells formed the basis for the research presented in this report, in which the focus moves from laboratory-based experiments to studies in an animal model.

Two types of skin “hypersensitivity” in mice were looked at. The first is a model of “delayed” or “contact” hypersensitivity [7-11], 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.

We investigated 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 [7]. The effects of histamine can be seen within minutes of the body's exposure to the allergy-inducing substance [8-9] and include an effect on nerves [10].

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.

The ultraviolet radiation in sunlight is responsible for sunburn, in which the redness and swelling (and sometimes blistering) of skin are indicative of an inflammatory response. The possibility that tea tree oil might be helpful in treating this type of inflammation was also investigated, again in a mouse model. However, no beneficial effect was found.

In summary, tea tree oil reduced the skin swelling found in a range of skin inflammatory reactions. This study provides scientific evidence for anecdotal reports that tea tree oil is useful in treating inflammatory reactions when applied topically to skin. Together with earlier laboratory-based studies, the results are an excellent foundation for more detailed studies of skin inflammation and itch in humans, and this research is underway.

Chapter 1. Introduction

Tea tree oil (TTO) is the essential oil steam-distilled from the Australian native plant, *Melaleuca alternifolia.* It is a complex mixture composed of approximately 100 monoterpene and sesquiterpene hydrocarbons and alcohols. The antimicrobial properties of TTO are well established [1-3]. However, anecdotal evidence together with recent scientific studies [4, 5] suggest TTO also has potential for use as a topical anti-inflammatory agent. Our earlier work showed that TTO could have direct anti-inflammatory effects on human white blood cells in studies using defined laboratory-based assays [4,5]. Other investigators, evaluating the properties of certain essential oils in rats, have shown that components of TTO, including α-pinene, α-terpinene, terpinen-4-ol, α-terpineol and linalool can reduce carrageenan-induced hind paw inflammation [20, 21]. However the activity of TTO *in vivo*, and mechanisms of the anti-inflammatory effects of TTO or any of its components, remain undefined. In this report, we present evidence that TTO can be beneficial in some models of inflammation in mice.

Effect of TTO on a chemical hypersensitivity response

Some humans develop skin sensitivities to certain chemicals (such as nickel, which can affect up to 10% of the population), in a reaction known as a contact hypersensitivity (CHS) response. This inflammatory reaction is mediated by immune lymphocytes following application of a low molecular weight chemical (hapten) to the skin, and later challenge with the same chemical. After sensitization, re-exposure to the same chemical results in a strong inflammatory response at the challenge site, a response which is much more vigorous than in an unsensitised individual [7-11]. We used a model of contact hypersensitivity in mice to evaluate the ability of TTO to regulate this type of inflammatory response. The components of TTO were also examined for their efficacy in this system.

Effect of TTO on the response of skin to ultraviolet irradiation

This model involves exposure of skin to ultraviolet B (UVB) radiation, which causes erythema (redness) and oedema (swelling) which peaks about 24 hours after exposure. A complex array of molecules, especially interleukin-10, tumour necrosis factor α (TNFα), prostaglandins, histamine, neuropeptides and reactive oxygen species, are involved in the response [12-15].

Effect of TTO on an allergic type of hypersensitivity

Allergic reactions, such as those seen in some individuals to certain foods (e.g., nuts, mushrooms), insect bites (e.g. bee stings) and pollens, can be vigorous, even life-threatening, within minutes of exposure to the inducing substance or allergen. Central to the body's response are mast cells, found in the skin, airways, and elsewhere in the body. In an allergic reaction, mast cells release a range of chemical mediators in response to allergens, in particular histamine [16]. This histamine will cause blood vessels to dilate (causing a reddish flare) and will increase the permeability of small blood vessels leading to plasma leakage and tissue swelling manifest as a wheal [17, 18], as well as to nerve fibre stimulation [19].

In this study, a potential therapeutic role for TTO in skin allergies was examined by investigating whether TTO affected histamine-induced ear swelling in mice. The murine model also allowed us to investigate the components of TTO responsible for the suppression of histamine-induced skin oedema.

Chapter 2: Tea tree oil reduces the swelling associated with the efferent phase of a contact hypersensitivity response

2.1 Materials and methods

Tea tree oil and its purified components

TTO was kindly provided by Thursday Plantation Laboratories Ltd (Ballina, NSW, Australia) and fulfilled the criteria of the Australian Standard [22] with a terpinen-4-ol level greater than 30% and 1,8-cineole level less than 15% as determined by gas chromatography-mass spectrometry. TTO was also supplied as a 5% ointment (containing 94% paraffin, wax, a smoothing ingredient and vitamin E) together with a base (TTO-free) control ointment. The water-soluble components of TTO were obtained as previously described [4, 5]. Briefly, TTO preparations of 1.25% were prepared in serum-free culture medium, mixed well and left to stand for 30 min. Gas chromatography/mass spectrometry confirmed that the oil soluble components adhered to the side of polystyrene plastic tubes whilst the water soluble components remained in the culture medium [4]. For individual study, terpinen-4-ol and α-terpineol were obtained from Fluka, Buchs, Switzerland and 1,8-cineole from Sigma Chemical Co., St Louis, MO.

Animals

Pathogen-free, female BALB/c mice were obtained from the Animal Resource Centre of the South Australian Department of Agriculture, Adelaide, Australia. The animals were maintained in an environment where ambient light was regulated on a twelve-hour light/dark cycle. The temperature in the room was controlled between 20-24°C with 50% humidity. Mice used for experiments ranged from 8-12 weeks of age. All experiments were performed according to the ethical guidelines of the National Health and Medical Research Council of Australia.

Assay of a contact hypersensitivity (CHS) response

BALB/c mice were sensitized on the shaved abdominal skin with 100 μl of freshly prepared 5% 2,4,6-trinitrochlorobenzene (TNCB; Kasei Kogyo Ltd., Tokyo, Japan) in acetone. Seven days later, the double skin fold thickness measured along the midline of a uniform dorsal area (8 cm^2, clean shaven 16-18 h prior) was evaluated using a micrometer (Mitutoyo Corp., Tokyo, Japan) before a CHS response was elicited by applying 50 μl of freshly prepared 1% TNCB in acetone to the shaved area (challenge phase). TTO (20 μl of undiluted; 30 μl of 5% ointment), TTO-free control ointment (30 μl), the water-soluble components of TTO (20 μl of 42% v/v terpinen-4-ol, 3% v/v α-terpineol and 2% v/v 1,8-cineole) or ethanol (20 μl) were applied topically to the shaved area at various time points before or after challenge and the change in double skin fold thickness was measured 3, 5, 7, 16, 24, 32, 40, 48 and 120 h later. The change in double skin fold thickness was calculated by subtracting the double skin fold thickness before challenge. The controls included mice that were treated with TTO alone

(sensitized 7 days prior, but not challenged with TNCB) and mice that were not sensitized 7 days prior but were challenged with TNCB.

Haematoxylin and Eosin (H and E) Staining

Samples of dorsal skin were rolled, fixed in 10% buffered formalin, paraffin embedded with a vertical orientation of the scrolls, and 4 μm sections were stained with Lillie Mayer's Haematoxylin for 4 min, followed by sequential rinses in acid alcohol and lithium carbonate. The sections were counterstained with Eosin for 2 min then rinsed in ethanol and xylene.

Adhesion molecule expression by Human Umbilical Vein Endothelial Cells (HUVECs)

HUVECs (4×10^5 cells) were incubated with TNFα (0.4 ng/ml, R&D Systems Inc, Minneapolis, MN) for 4 h at 37°C for induction of E-selectin expression. The water-soluble components of TTO (0.125% v/v) were added 60 min or 30 min before TNFα or 2 h afterwards. The supernatant was removed and the cell monolayers washed with 10 mM EDTA in PBS before detachment using trypsin-EDTA. The cells were pelleted (2000 rpm for 2 min at 4°C), the supernatant aspirated and 100 μl anti-E selectin (49-IB11 prepared in-house) or isotype control antibody added for 30 min at 4°C. The cells were pelleted, the supernatant aspirated and 100 μl FITC-conjugated anti-mouse antibody (Silenus Laboratories, Hawthorn, Australia) added for 30 min at 4°C. After washing, the cells were resuspended in FACSfix (phosphate buffered saline supplemented with 1% formaldehyde, 2% glucose and 5 mM sodium azide) prior to flow cytometric analysis on FACScan (Becton Dickinson, Mountain View, CA).

UV irradiation of mice

The UV source was a bank of FS40 sunlamps (Westinghouse Corp., Pittsburgh, PA) emitting a broad band of UV, 250-360 nm, with 65% of the output in the UVB range (280-320 nm). A polyvinylchloride plastic sheet was used to screen out wavelengths < 290 nm. The dose rate was monitored using a UVX radiometer with a UVX-31 sensor (Ultraviolet Products Inc., San Gabriel, CA). For irradiation of mice, the double skin fold thickness along the midline of a uniform dorsal area (8 cm^2, clean shaven 16-18 h prior) was measured and the mice were housed in individual compartments of a perspex cage which was placed 20 cm below the UV source before being exposed to the appropriate UVB dose (8 or 2 kJ/m^2). TTO (20 μl of undiluted) was applied topically to the shaved area at various time points before or after UVB exposure and the change in double skin fold thickness was measured 24, 48 and 120 h later. The change in double skin fold thickness was calculated by subtracting the skin thickness before UVB exposure. The controls included mice that were treated with TTO alone but not exposed to UVB.

Expression of results and statistical analysis

A multiple comparison procedure employing a one-way analysis of variance and Fisher's test was used to determine the statistical significance of differences among mice within CHS and UVB experiments. The data represent change in double skin fold thickness (mean + SEM) of all mice that were tested in a similar manner (3 mice per group for up to 5 independent experiments). Probabilities less than 0.05 were considered significant.

2.2 Results

The effect of TTO on the swelling associated with the efferent phase of a CHS response

To investigate the time course of the CHS response, a change in double skin fold thickness at the hapten-treated site was measured at various time points after TNCB challenge. BALB/c mice were sensitized epicutaneously on abdominal skin with TNCB and challenged on dorsal skin 7 days later. Alternatively, mice that were not sensitized 7 days prior were challenged only with TNCB (Fig 2.1). For the first 7 h post challenge, swelling was detected in the skin of both sensitized mice and non-sensitized mice. The change in double skin fold thickness in the non-sensitized mice (irritant response) subsided significantly in the following 17 h whilst it remained high in the sensitized mice. Undiluted TTO applied 30 min prior to TNCB application to the non-sensitized mice was unable to reduce the increase in double skin fold thickness observed in the first 7 h after TNCB exposure (data not shown).

Subsequently for all experiments, the change in double skin fold thickness was measured 24, 48 and 120 h post TNCB challenge to sensitized mice. For each of 5 independent experiments, mice that received a single topical application of undiluted TTO 30 min before challenge, or 2, 4 or 7 h after challenge showed significantly reduced swelling (Fig 2.2). In fact, if TTO was applied 4 h after challenge the swelling induced was not significantly different from the swelling in mice exposed to TTO in the absence of TNCB challenge at all time points measured.

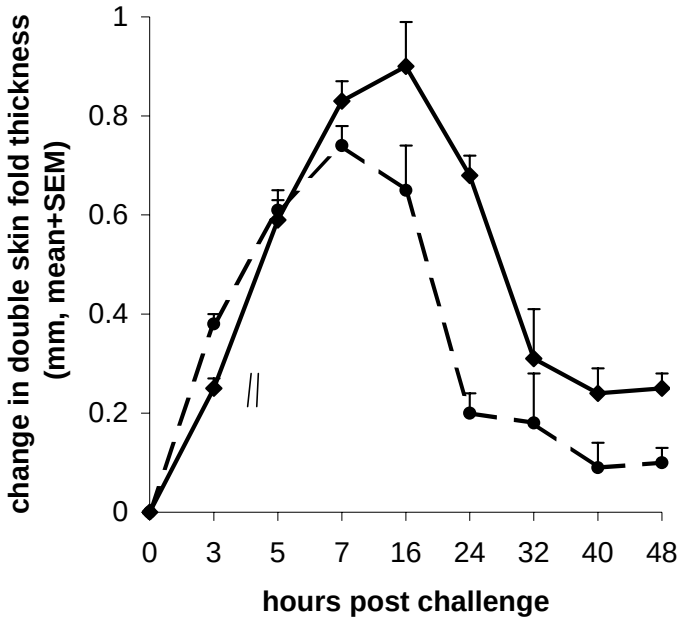

Figure 2.1. Time course of the swelling associated with the efferent phase of a CHS response. BALB/c mice were sensitized by application of TNCB on abdominal skin and challenged on dorsal skin with TNCB 7 days later (full line). Alternatively, BALB/c mice were not sensitized but only challenged with TNCB (broken line). A change in double skin fold thickness was measured 3, 5, 7, 16, 24, 32, 40, and 48 h post challenge; mean + SEM of 6 mice (3 mice per group for 2 independent experiments).

The effect of a 5% TTO ointment and its TTO-free control ointment on TNCB-induced swelling was investigated. The ointments were applied as a single topical application 7 h after challenge. The control ointment had minimal effect, whereas the 5% TTO ointment significantly suppressed TNCB-induced swelling by 39%. Neither ointment caused significant swelling in the absence of hapten challenge (Fig 2.3).

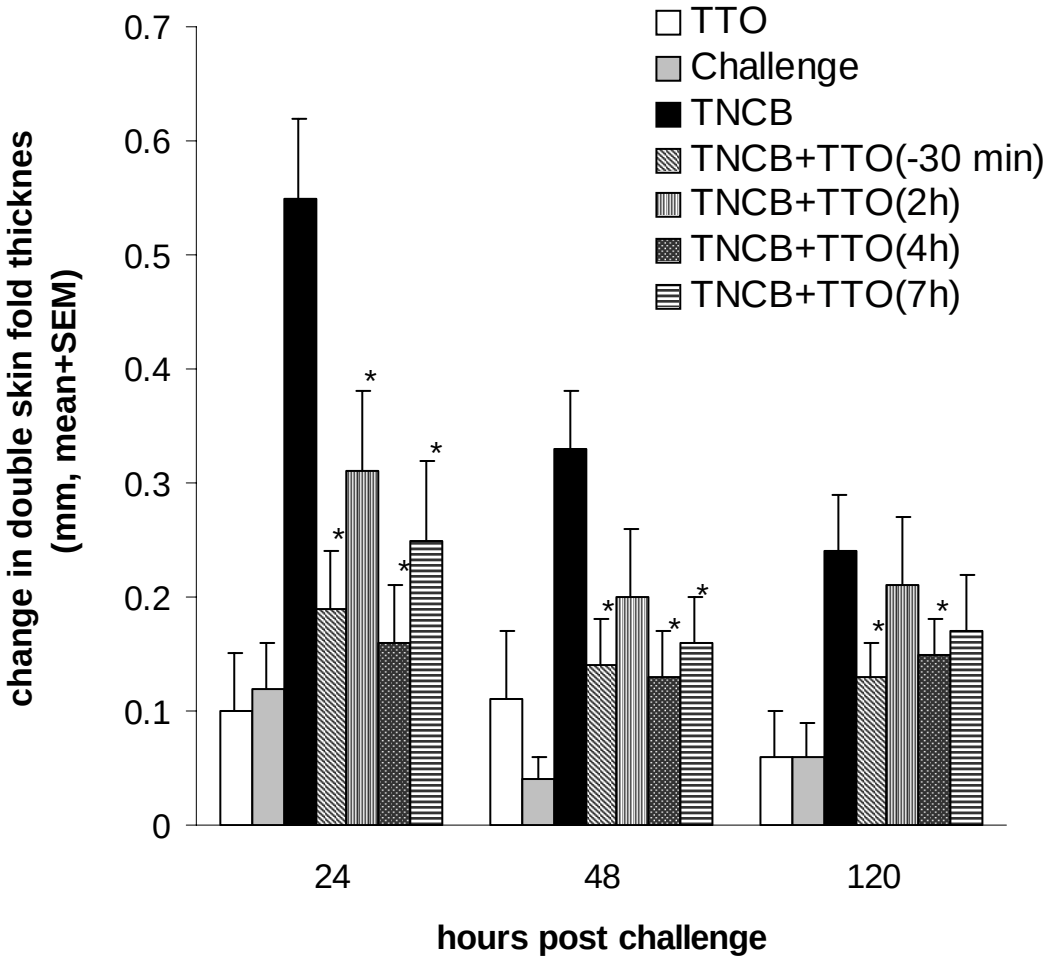

Figure 2.2. The effect of topical application of undiluted TTO on the swelling associated with the efferent phase of a CHS response. BALB/c mice were sensitized by application of TNCB on abdominal skin and challenged on dorsal skin with TNCB 7 days later. Undiluted TTO was administered to the challenge site as a single application 30 min before, or 2 h, 4 h, or 7 h after TNCB challenge and the change in double skin fold thickness was measured 24, 48 and 120 h post challenge. Mice that were not sensitized, but only challenged with TNCB (Challenge), showed negligible swelling. The data represent change in double skin fold thickness (mean + SEM) of 15 mice (3 mice per group for 5 independent experiments). An asterisk indicates a significant suppression by TTO of TNCB-induced swelling.

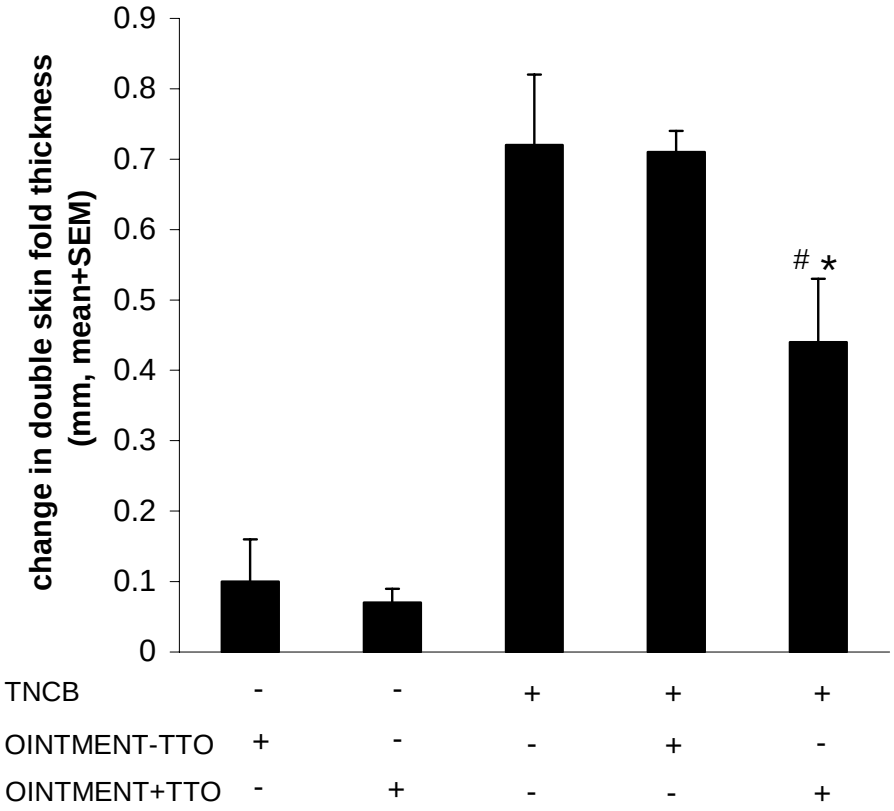

Figure 2.3. The effect of topical application of 5% TTO on the swelling associated with the efferent phase of a CHS response. BALB/c mice were sensitized by application of TNCB on abdominal skin and challenged on dorsal skin with TNCB 7 days later. A 5% TTO ointment (OINTMENT+TTO) or control ointment (OINTMENT-TTO) was administered as a single application in the absence of challenge (-TNCB) or 7 h after challenge (+TNCB). The change in double skin fold thickness was measured 24 h post challenge; mean + SEM of 6 mice (3 mice per group for 2 independent experiments). Ointments alone caused minimal swelling in the absence of hapten challenge. An asterisk (*) indicates a significant suppression by TTO of TNCB-induced swelling. A hash (#) indicates a significant difference in TNCB-induced swelling by TTO ointment compared to control ointment.

The effect of TTO on skin pathology in vivo

To further elucidate whether the suppressive effect of TTO on TNCB-induced swelling could be attributed to changes in the cellular infiltrate and/or oedema associated with inflammation, dorsal skin was sampled 7 or 24 h post TNCB challenge, formalin-fixed and examined by H and E staining and immunohistochemistry. As suggested by the measured increase in double skin fold thickness, skin collected 7 h after TNCB challenge demonstrated microscopic signs of swelling (Fig 2.4). There was an influx of inflammatory cells into the dermis and subcutaneous fatty tissue (SCFT) and an alteration in the width and structure of the SCFT (Fig 2.4C). The swelling due to TNCB application to non-sensitized skin was due mainly to increased fluid retention rather than an increased influx of inflammatory cells as the latter was not present in the section (Fig 2.4B).

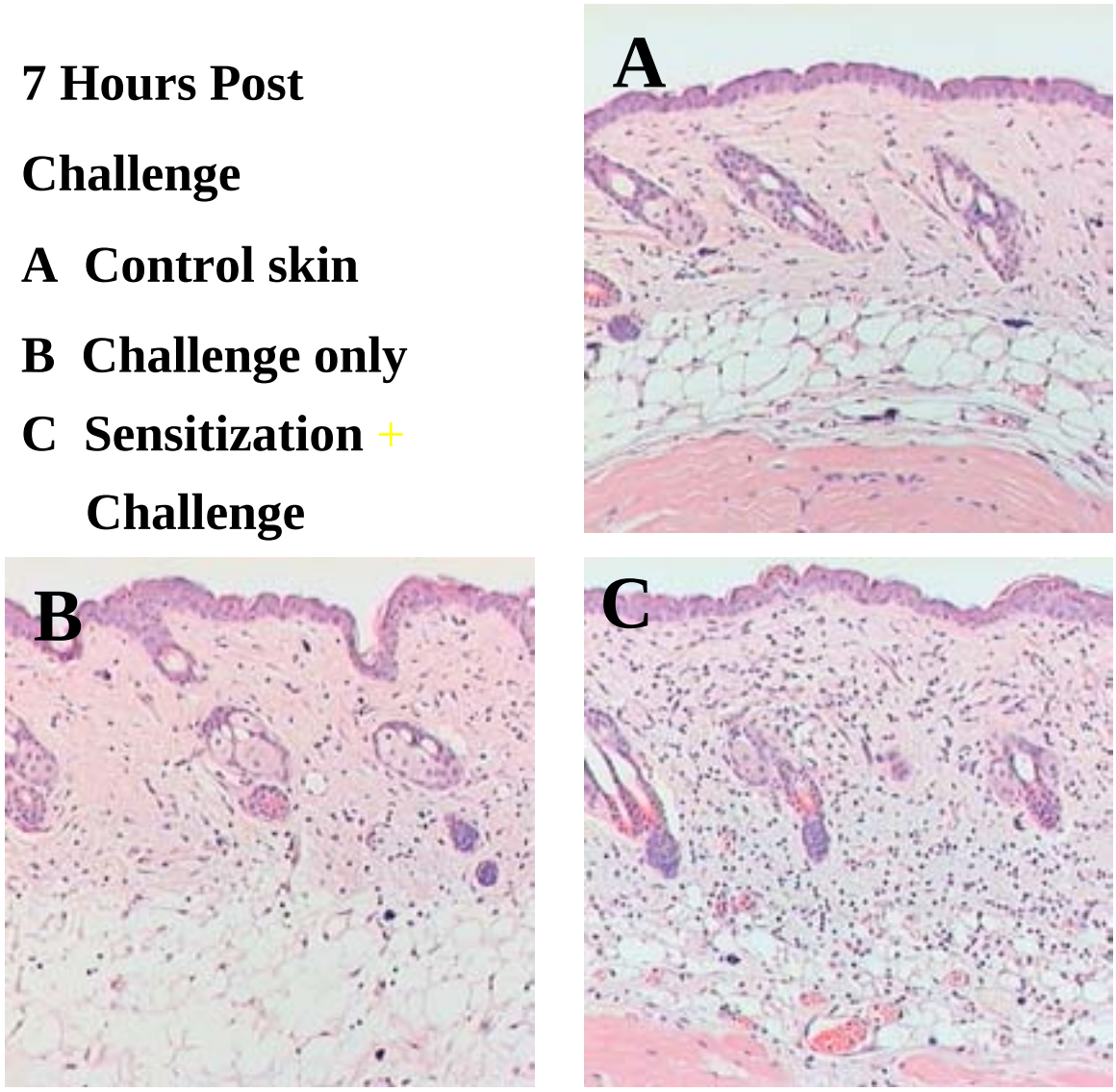

Figure 2.4. Histological sections of dorsal skin of mice 7 h after exposure to TNCB. A. Control skin. B. Skin of non-sensitized mice exposed to TNCB. C. Skin of TNCB-sensitized mice challenged with TNCB. All skins were formalin fixed, paraffin embedded and 4 μm thick sections stained with H and E. The epidermal layer is at the top of the figures, the SCFT at the bottom.

Skin collected 24 h after TNCB challenge to sensitized skin also showed extensive infiltration of inflammatory cells (including polymorphonuclear cells, confirmed by staining with an anti-neutrophil antibody (RB6-8C5, [23]) into the dermis and SCFT and loss of the normally regular, well-defined shape and structure of the SCFT. Skin collected 24 h after TNCB challenge to sensitized skin demonstrated that topical application of undiluted TTO 7 h after challenge, did not prevent the passage of infiltrating inflammatory cells into the dermis and SCFT, but resulted in less epidermal necrosis, less cytoclasis, less leucocytoclasis and retention of the well-defined shape and structure of the SCFT that was evident in control tissue (Fig 2.5). In the absence of TNCB exposure, topical application of undiluted TTO did not alter the pathology of dorsal skin tissue (data not shown).

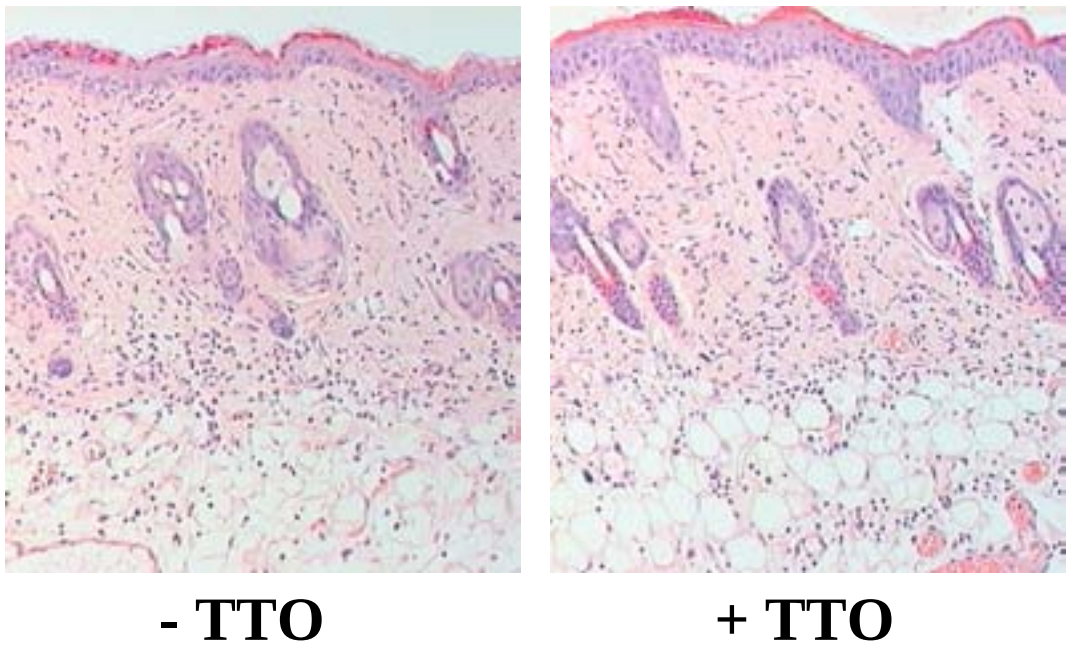

Figure 2.5. Histological sections of dorsal skin of TNCB-sensitized mice 24 h after subsequent challenge with TNCB, with or without application of undiluted TTO 7 hours after TNCB challenge.

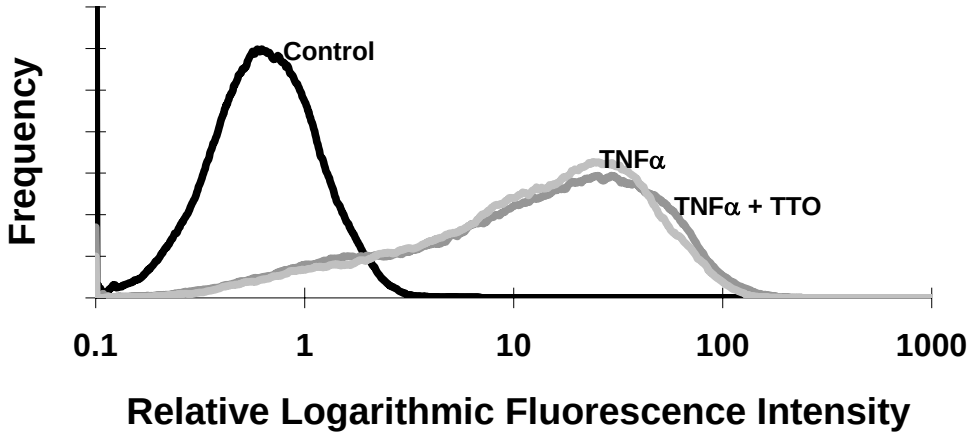

Figure 2.6. The effect of TTO on E-selectin expression by HUVECs. HUVECs (4 x 10^5 cells) were incubated with TNFα (0.4 ng/ml) for 4 h at 37°C for induction of E-selectin expression. The water-soluble components of TTO (0.125% v/v) were added 30 min before and during the incubation with TNFα. HUVECs were detached, pelleted and then incubated with anti-E selectin or isotype control antibodies. The control shows the relative logarithmic fluorescence intensity for HUVECs stained with the isotype control antibody. Incubation with TTO did not affect the staining of the isotype control antibody (data not shown).

The effect of TTO on E-selectin expression by HUVECs

The water-soluble components of TTO at a concentration of 0.125% had no inhibitory effect on E-selectin expression by control HUVECs, or by TNFα-stimulated HUVECs if added 30 min before TNFα (Fig 2.6), 60 min before TNFα or 2 h after TNFα (data not shown). This concentration of TTO had been shown previously to cause maximal regulatory effects on human monocytes *in vitro*, in the absence of toxicity [3].

The effect of the water-soluble components of TTO on the swelling associated with the efferent phase of a CHS response

The effect of the water-soluble components of TTO on TNCB-induced swelling was investigated. The water-soluble components (previously identified to be principally terpinen-4-ol, α-terpineol and 1,8-cineole, [3]) were applied at concentrations equivalent to those found in TTO (i.e. at 42%, 3% and 2% v/v, respectively) as a single topical application 4 h after challenge. The water-soluble components of TTO were diluted in ethanol to aid penetration through the skin. However, ethanol had no effect on swelling in the absence (data not shown) or presence of hapten challenge (Fig 2.7A and B).

When applied individually, only terpinen-4-ol reduced swelling significantly, and not to the same extent as seen with TTO; α-terpineol and 1,8-cineole were without significant effect (Fig 2.7A). When applied in combination, terpinen-4-ol together with α-terpineol significantly reduced swelling, to an extent similar to that seen with TTO (Fig 2.7B). The components did not cause significant swelling in the absence of hapten challenge (data not shown).

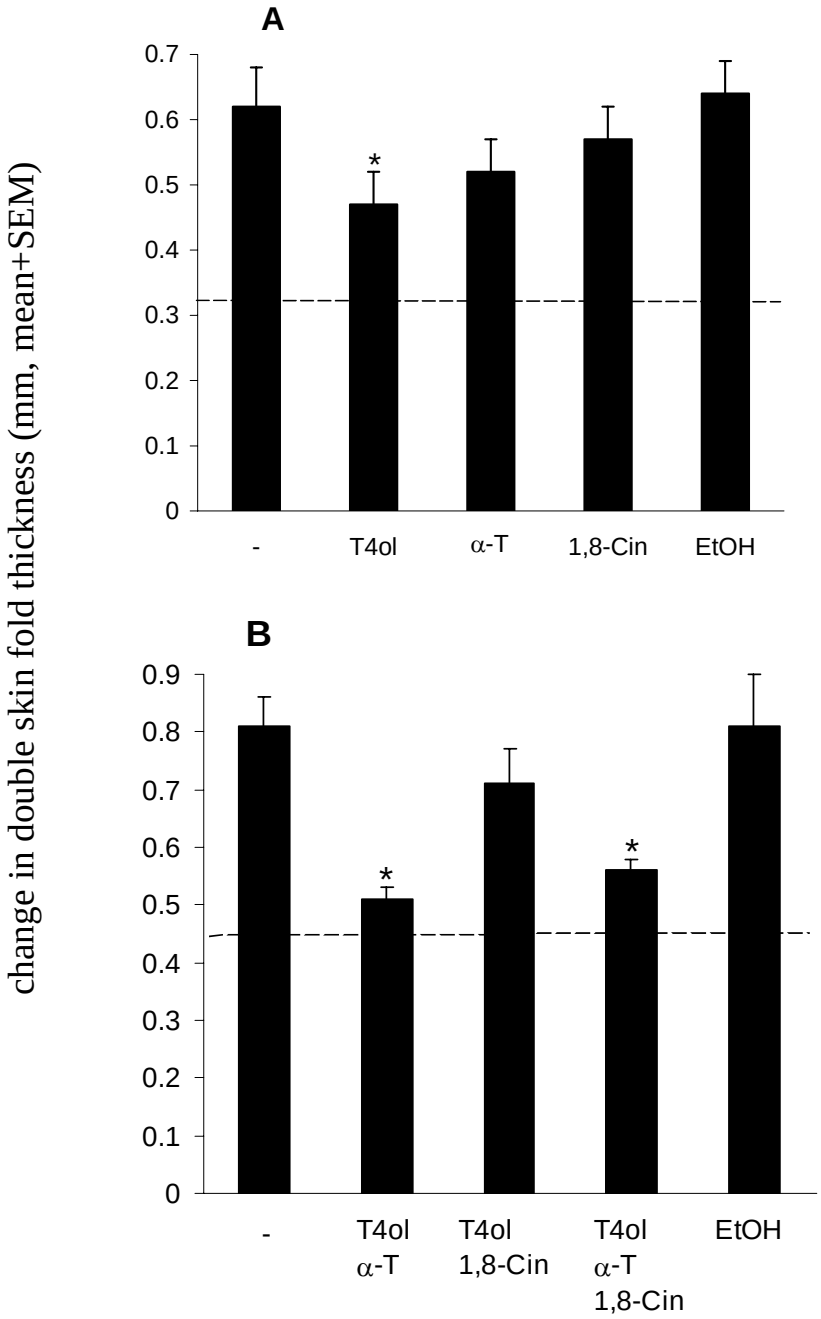

Figure 2.7. The effect of topical application of the water-soluble components of TTO on the swelling associated with the efferent phase of a CHS response. BALB/c mice were sensitized by application of TNCB on abdominal skin and challenged on dorsal skin with TNCB 7 days later. The water soluble components of TTO (terpinen-4-ol, T4ol; α-terpineol, α-T; 1,8-cineole, 1,8-Cin) or ethanol (EtOH) were administered alone to the challenge site (A), or in combination (B), as a single application 4 h after TNCB challenge. The change in double skin fold thickness was measured 24 h post challenge. The dotted line represents the suppression by undiluted TTO of TNCB-induced swelling. The data represent change in double skin fold thickness (mean + SEM) of up to 11 mice (2-3 mice per group for 4 independent experiments). An asterisk indicates a significant suppression by the water-soluble components of TTO of TNCB-induced swelling.

The effect of TTO on the swelling induced by UVB irradiation

UVB irradiation (8 kJ/m^2) of the shaved dorsal skin of naïve BALB/c mice caused an oedematous response after 24 h (Fig 2.8A). A single topical application of undiluted TTO was unable to suppress UVB-induced swelling regardless of whether it was applied before or after UVB exposure. The swelling induced by exposure to 8 kJ/m^2 UVB radiation was approximately 2-fold greater than that obtained through elicitation of a CHS response. Exposing mice to a lower dose of UVB radiation (2 kJ/m^2) induced an oedematous response comparable to that obtained following sensitization and challenge with TNCB (Fig 2.8B). However, TTO was still unable to suppress the UVB-induced swelling.

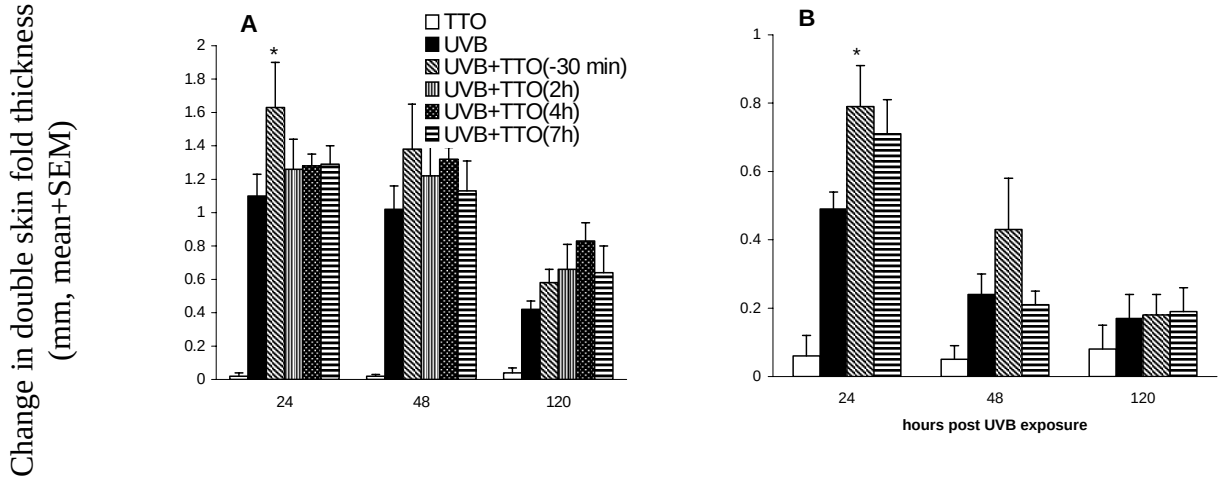

Figure 2.8. The effect of topical application of undiluted TTO on the swelling induced by UVB irradiation. The shaved dorsal skin of BALB/c mice was UVB-irradiated at a dose of 8 kJ/m^2 (A) or 2 kJ/m^2 (B). In A, undiluted TTO was administered as a single application 30 min before, or 2, 4, or 7 h after UVB exposure (8 kJ/m^2). The change in double skin fold thickness was measured 24, 48 and 120 h post UVB exposure; mean + SEM of 9 mice (3 mice per group for 3 independent experiments). In B, undiluted TTO was administered as a single application 30 min before, or 7 h after UVB exposure (2 kJ/m^2). The change in double skin fold thickness was measured 24, 48 and 120 h post UVB exposure; mean + SEM for 3 mice from a single experiment. An asterisk represents a significant increase in double skin fold thickness above that due to UVB irradiation.

2.3 Discussion

In this study, skin oedema developed in mice in response to three stimuli, presumably by three different pathways. Firstly, oedema developed by challenge of presensitized mice with TNCB. Secondly, there was a short-lived (<24 h) oedema due to the irritancy of TNCB on skin of mice not previously exposed to that chemical. Thirdly, UVB caused oedema at irradiated sites. Only the oedema developing during a specific immune response was reduced by application of TTO. When applied topically to a pre-sensitized mouse 30 min before or up to 7 h after challenge with the sensitizing antigen, TTO significantly curtailed the development of CHS-associated swelling. In contrast, TTO was without effect on the swelling measured in skin of non-sensitized mice exposed to the experimental hapten, TNCB. In addition, regardless of whether TTO was applied before, or up to 7 h after exposure to UVB, no significant effect was seen on the inflammatory oedema. These results suggest that

memory T cells produce, or otherwise regulate, the production of a factor(s) that modulates the oedema associated with a CHS response and this process can be regulated by TTO.

For TNCB-sensitized mice, dorsal skin measured 7 h after TNCB challenge showed obvious thickening. H and E staining demonstrated that even at this early time point, there was an influx of inflammatory cells into the dermis and SCFT and an alteration in the structure of the SCFT. Interestingly, if TTO was applied topically up to 7 h after hapten challenge it did not reduce the influx of inflammatory cells induced by antigenic challenge, but was still able to regulate the TNCB-induced swelling at 24 h. This would suggest TTO controls the development of oedema by a process not associated with the influx of cells *per se* into the tissue. As significant swelling was also detected in the skin of non-sensitized mice exposed to TNCB for 7 h and TTO did not reduce this swelling, TTO controls the hapten-specific phase of swelling in the pre-sensitized mice. Expression of E-selectin was examined as it is an endothelial cell molecule that mediates adhesion between circulating leukocytes and endothelial cells, a first step for leukocytes before being activated and transmigrating into the tissue during inflammation. Flow cytometric analysis demonstrated the addition of the water-soluble components of TTO to unstimulated or TNFα-stimulated HUVECs did not reduce E-selectin expression. These results further suggest the suppressive effect of TTO on TNCB-induced swelling is not due to a reduction in influx of inflammatory cells but to a reduction in oedema. As unmyelinated c-type sensory fibres in the skin are involved in regulation of cutaneous inflammatory reactions through release of neuropeptides such as tachykinins (substance P, neurokinins) and calcitonin gene-related peptide [24], we are currently investigating the role of neuropeptides in the regulation by TTO of the oedema associated with recall immune responses.

A small proportion of the human population will experience adverse skin reactions (irritant or allergic contact dermatitis) following topical application of undiluted, but not 10% TTO [25, 26]. Application of a 5% TTO-containing ointment significantly suppressed TNCB-induced swelling 24 h after hapten challenge to sensitized skin. Similar experiments were conducted using a 10% TTO-containing gel (prepared in 80% ethanol with a thickening agent incorporated). However the TTO-free control gel significantly suppressed TNCB-induced swelling, and the superimposed suppressive effect seen with incorporation of TTO did not reach significance (data not shown). In reality, an individual may topically administer TTO to a skin lesion more than once. As a single application of 5% TTO significantly reduced the swelling associated with an immune response, we hypothesize that multiple applications may augment this suppression. Lower concentrations of TTO will also reduce the risk of irritant skin reactions occurring following topical application. This study was performed with TNCB and mice. Many individuals suffer CHS responses upon re-exposure to plant and insect antigens. Nickel is also a frequent contact allergen in the general population [27]. We hypothesize that TTO may reduce the inflammation associated with such hypersensitivity responses.

In vitro studies have shown that unfractionated TTO is toxic to mammalian cells [28, 29], but that the water-soluble components of TTO can regulate inflammatory mediator production by activated human peripheral blood monocytes, in the absence of toxicity [4, 5]. Furthermore, the *stratum corneum* may act as a selective barrier differentially retaining the hydrophobic components and allowing penetration of the water-soluble components into the dermis. TTO is also known to contain components, in particular terpenes that enhance percutaneous drug absorption [30-32] and would therefore enable penetration of the active components of TTO through the epidermis into the dermal blood supply allowing for regulation of an inflammatory response. The results of this study demonstrate that the suppression by TTO of TNCB-induced swelling may be attributable to the actions of the TTO components, terpinen-

4-ol and α-terpineol. However, further work evaluating the identity and concentration of TTO components that can penetrate through the epidermis *in vivo* need to be performed.

2.4 Conclusion

Together with our previous investigations [4, 5], this study continues the unravelling of the biological responses that follow the exposure of skin to TTO. We have demonstrated that TTO can regulate the swelling associated with an inflammatory response to a chemical that induces a contact hypersensitivity response, but is ineffective in controlling the oedema associated with non-specific inflammation following UVB or exposure of non-sensitized skin to an irritant. As more scientific evidence is obtained and the mechanism of the anti-inflammatory activity of TTO recognized, the rationale of its use for treatment of inflammatory reactions chemicals that induce hypersensitivity will be increased.

Chapter 3. Tea tree oil reduces histamine-induced oedema in murine ears

3.1 Materials and methods

Tea tree oil and its purified components

TTO was kindly provided by Thursday Plantation Laboratories Ltd (Ballina, NSW, Australia) and fulfilled the criteria of the Australian Standard [22] with a terpinen-4-ol level greater than 30% and 1,8-cineole level less than 15% as determined by gas chromatography-mass spectrometry. TTO was also supplied as a 5% ointment (containing 94% paraffin, wax, a smoothing ingredient and vitamin E) together with a base (TTO-free) control ointment. The water-soluble components of TTO were obtained as previously described [4, 5]. Briefly, TTO preparations of 1.25% were prepared in serum-free culture medium, mixed well and left to stand for 30 min. Gas chromatography/mass spectrometry confirmed that the oil soluble components adhered to the side of polystyrene plastic tubes whilst the water soluble components remained in the culture medium [4]. For individual study, terpinen-4-ol and α-terpineol were obtained from Fluka, Buchs, Switzerland and 1,8-cineole from Sigma Chemical Co., St Louis, MO.

Animals

Pathogen-free, female BALB/c mice were obtained from the Animal Resource Centre of the South Australian Department of Agriculture, Adelaide, Australia. The animals were maintained in an environment where ambient light was regulated on a twelve-hour light/dark cycle. The temperature in the room was controlled between 20-24°C with 50% humidity. Mice used for experiments ranged from 8-12 weeks of age. All experiments were performed according to the ethical guidelines of the National Health and Medical Research Council of Australia.

Histamine induction of oedema

Mice were anaesthetized (ketamine 75 mg/kg and medetomidine 1 mg/kg intraperitoneally) prior to measuring the thickness of the ears using a spring loaded micrometer (Mitutoyo Corp., Tokyo, Japan). Histamine diphosphate (Sigma, 10 μl of solutions of 10-100 mg/ml in 0.9% saline) was injected intradermally into the dorsal aspect of each ear of a mouse using an insulin syringe. Both ears of a control mouse were injected with an equal volume of saline (0.9% solution; Baxter Healthcare Pty Ltd, NSW, Australia). Undiluted TTO (10 μl) was topically administered to both sides of the right ear of each mouse, 30 min before or immediately after histamine or saline injection. Alternatively, 10 μl of a solution of terpinen-4-ol (20% to 100% v/v in ethanol), 10 μl of a 3% v/v solution of α-terpineol in ethanol or 10 μl of a 2% v/v solution of 1,8-cineole in ethanol, were topically administered to both sides of the right ear of each mouse, immediately after histamine or saline injection. Ethanol (100%) was used as the control. In one experiment, different volumes of terpinen-4-ol were applied to the test ear. The left ear received no further treatment. Histamine- or saline-induced swelling of the ears was then measured every 15 min for the next 90 min.

Neuropeptide depletion of sensory c-fibres in BALB/c mice

Neuropeptide activity of sensory c-fibres was depleted in BALB/c mice as previously described [33]. Briefly, BALB/c mice at 4 weeks of age were anaesthetized (see above) and injected subcutaneously in the neck region with 50 μl capsaicin (25 mg/kg; Sigma) or vehicle control (alcohol:Tween80:saline; 2:1:7) on two consecutive days. After completion of studies of histamine-induced oedema performed on the mice when 8 weeks of age, neuropeptide depletion was confirmed [33].

Expression of results and statistical analysis

The data represent ear swelling (mm) of all ears that were tested in a similar manner. A multiple comparison procedure comparing a one-way analysis of variance and Fischer's test was used to determine the statistical significance of differences in measurements of ear swelling. Probabilities less than 0.05 were considered significant.

3.2 Results

The effect of topical administration of TTO to murine ears

The effect of topical administration of undiluted TTO to both sides of murine ears was investigated. TTO caused minimal ear thickening. Fifteen min after administration, the mean change ($\pm$ SD) in ear swelling above baseline was 0.02 $\pm$ 0.01 mm for BALB/c mice (n=6) and 0.01 $\pm$ 0.01 mm for C57BL/6 mice (n=3). The latter strain of mouse has a higher mast cell prevalence in dorsal skin [34]. Mean ear swelling increased to 0.05 and 0.06 mm, respectively, 45 min after administration, before declining (Figure 3.1 shows data for BALB/c mice).

The effect of topical administration of TTO on the oedematous response induced by saline injection in murine ears

Intradermal injection of saline into the dorsal aspect of the ears of BALB/c mice resulted in non-specific swelling that peaked 15 min after injection, but declined thereafter (Figure 3.1). TTO significantly increased saline-induced ear swelling if administered to BALB/c mice 30 min before, or immediately after, intradermal injection of saline (Figure 3.2A). TTO applied immediately after saline injection also significantly increased the swelling induced in C57BL/6 mice (Figure 3.2B).

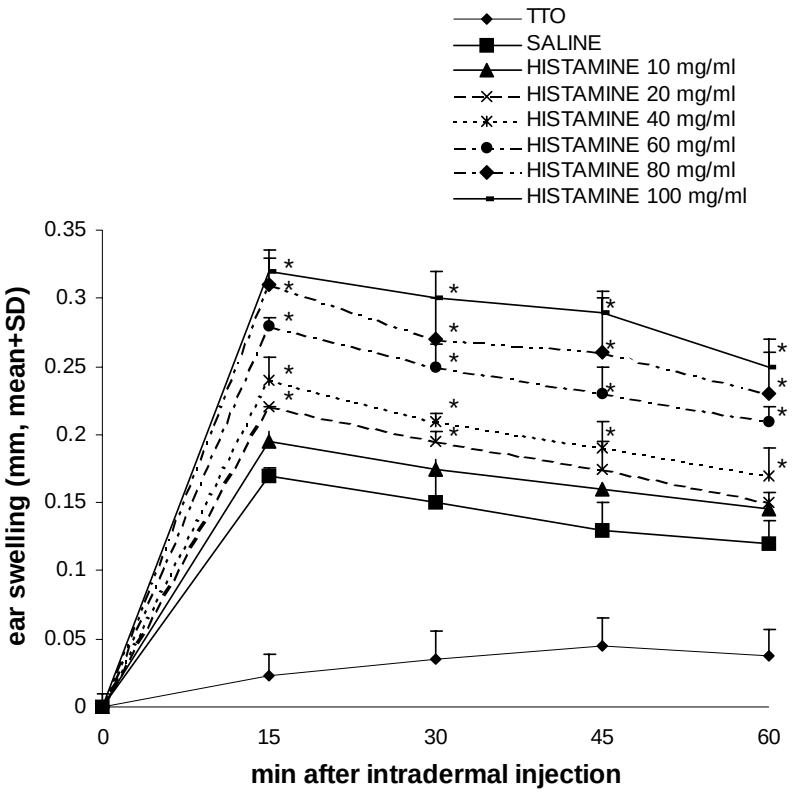

Figure 3.1. The effect of intradermal histamine injection on ear swelling in BALB/c mice. Ten μl of saline or histamine diphosphate (10, 20, 40, 60, 80 or 100 mg/ml solution in 0.9% saline) was injected intradermally into the dorsal aspect of both ears of a mouse using an insulin syringe. Saline or histamine-induced swelling of the ears was then measured every 15 min for the next 60 min. Alternatively, TTO alone (TTO) was topically administered to both sides of each ear of a mouse and the swelling measured every 15 min for the next 60 min. The data represent ear swelling (mm, mean+SD) of 3 ears.

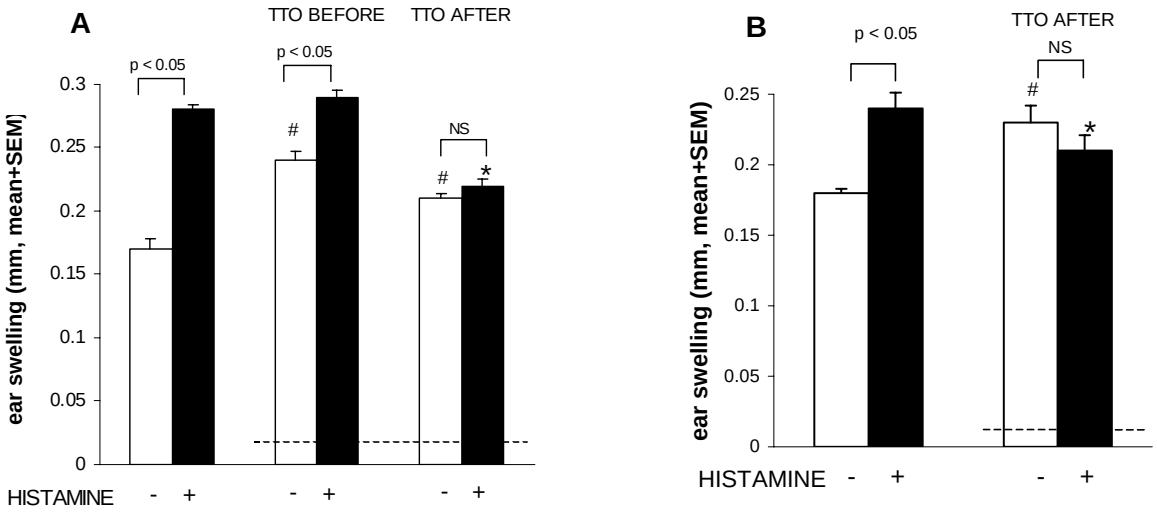

Figure 3.2. The effect of topical administration of TTO on the oedematous response induced by saline and histamine injection in the ears of A. BALB/c, and B. C57BL/6 mice. Ten µl of saline or histamine diphosphate (60 mg/ml solution in 0.9% saline) was injected intradermally into the dorsal aspect of both ears of a mouse using an insulin syringe. In A, undiluted TTO was topically administered to both sides of the right ear of each mouse, 30 min before (TTO before) or immediately after (TTO after) saline (-) or histamine (+) injection. The left ear received no further treatment. The data represent ear swelling (mm, mean+SEM) 15 minutes after intradermal injection of 12 ears (3 ears per group for 4 independent experiments). In B, undiluted TTO was topically administered to both sides of the right ear of each mouse, immediately after (TTO after) saline (-) or histamine (+) injection. The left ear received no further treatment. The data represent ear swelling (mm, mean+SEM) 15 min after intradermal injection of 3 ears per group. A hash (#) indicates a significant increase by TTO of saline-induced swelling. An asterisk (*) indicates a significant suppression by TTO of histamine-induced swelling. The dotted line shows the swelling after 15 min of non-injected skin treated with TTO.

The effect of topical administration of TTO on the oedematous response induced by histamine injection in murine ears

Intradermal injection of histamine into the dorsal aspect of the ears of BALB/c mice resulted in swelling that was significantly higher than that obtained with saline injection (Figure 3.1, 3.2A). The effect of histamine was dose-dependent with a significant change measured at concentrations greater than, or equal to 20 mg/ml (Figure 3.1). Histamine-induced swelling was maximal after 15 min (Figure 3.1). Intradermal injection of a 60 mg/ml solution of histamine was used for all subsequent experiments.

If undiluted TTO was topically administered 30 min before histamine injection to BALB/c mice, histamine still induced significant ear swelling. In contrast, TTO applied immediately after histamine injection significantly reduced the oedematous effect of histamine to the extent that there was no difference between TTO-treated saline and TTO-treated histamine-injected ears (Figure 3.2A).

A similar response to TTO was detected in the ears of C57BL/6 mice. Against a background of TTO-induced swelling in saline-injected ears, TTO applied immediately after histamine injection significantly reduced histamine-induced ear swelling (Figure 3.2B).

The effect of topical administration of TTO on the oedematous response induced by histamine injection in ears of sensory neuropeptide-depleted BALB/c mice

BALB/c mice were treated with capsaicin when 4 weeks of age to deplete sensory neuropeptides [33]. For both capsaicin and non-capsaicin-treated mice, topical administration of TTO to both sides of the ear immediately after histamine injection, significantly reduced ear swelling to levels less than that seen in TTO-treated, saline-injected ears (Figure 3.3).

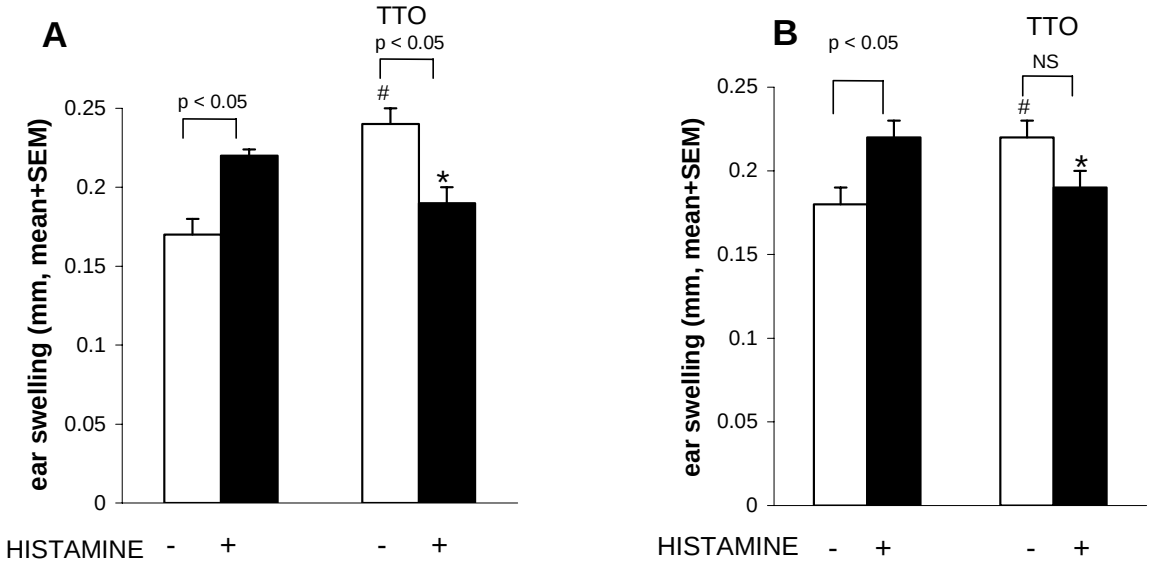

Figure 3.3. The effect of topical administration of TTO on the oedematous response induced by saline and histamine injection in the ears of sensory neuropeptide-depleted BALB/c mice. Ten μl of saline or histamine diphosphate (60 mg/ml solution in 0.9% saline) was injected intradermally into the dorsal aspect of both ears of A. control mice, and B. sensory neuropeptide-depleted mice. Undiluted TTO was topically administered to both sides of the right ear of each mouse immediately after saline (-) or histamine (+) injection. The left ear received no further treatment. The data represent extent of ear swelling (mm, mean+SEM) 15 min after intradermal injection of 9 ears (3 ears per group for 3 independent experiments). A hash (#) indicates a significant increase by TTO of saline-induced swelling. An asterisk (*) indicates a significant suppression by TTO of histamine-induced swelling.

The effect of topical administration of the water-soluble components of TTO on the oedematous response induced by saline and histamine injection in BALB/c ears

The effects of the major water-soluble components of TTO on saline- and histamine-induced ear swelling were investigated. The water-soluble components were prepared in ethanol to aid penetration through the skin. However, ethanol had no effect on saline- or histamine-induced ear swelling (Figure 3.4).

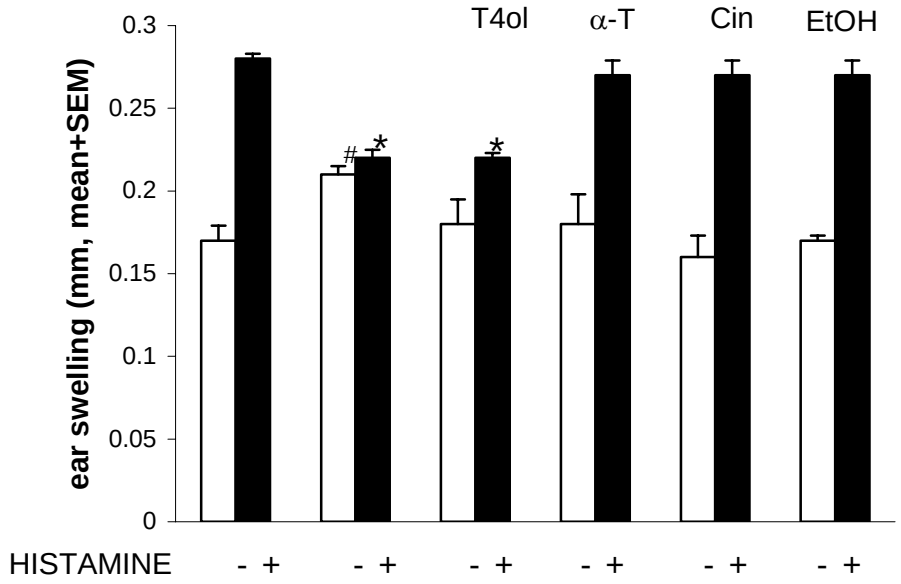

Figure 3.4. The effect of topical administration of the water-soluble components of TTO on the oedematous response induced by injection of saline and histamine injection in BALB/c ears. Ten μl of saline or histamine diphosphate (60 mg/ml solution in 0.9% saline) was injected intradermally into the dorsal aspect of both ears of a mouse. TTO, terpinen-4-ol (T4ol), α-terpineol (α-T), 1,8-cineole (Cin) or the appropriate ethanol control (EtOH) was topically administered to both sides of the right ear of each mouse immediately after saline (-) or histamine (+) injection. The left ear received no further treatment. The data represent ear swelling (mm, mean+SEM) 15 min after intradermal injection of 3 ears. A hash (#) indicates a significant increase by TTO of saline-induced swelling. An asterisk (*) indicates a significant suppression by TTO of histamine-induced swelling.

When diluted in ethanol to concentrations equivalent to those found in TTO (i.e. terpinen-4-ol, α-terpineol and 1,8-cineole at 42%, 3% and 2%, respectively) and in direct contrast to the effect of TTO, there was no significant effect on basal or saline-induced ear swelling (Figure 3.4). Only terpinen-4-ol reduced histamine-induced ear swelling; α-terpineol and 1,8-cineole were without significant effect (Figure 3.4). The suppressive effect of terpinen-4-ol was significant and dose-dependent regardless of whether it was administered at increasing concentrations in a constant volume (Figure 3.5A), or undiluted in increasing volumes (Figure 3.5B).

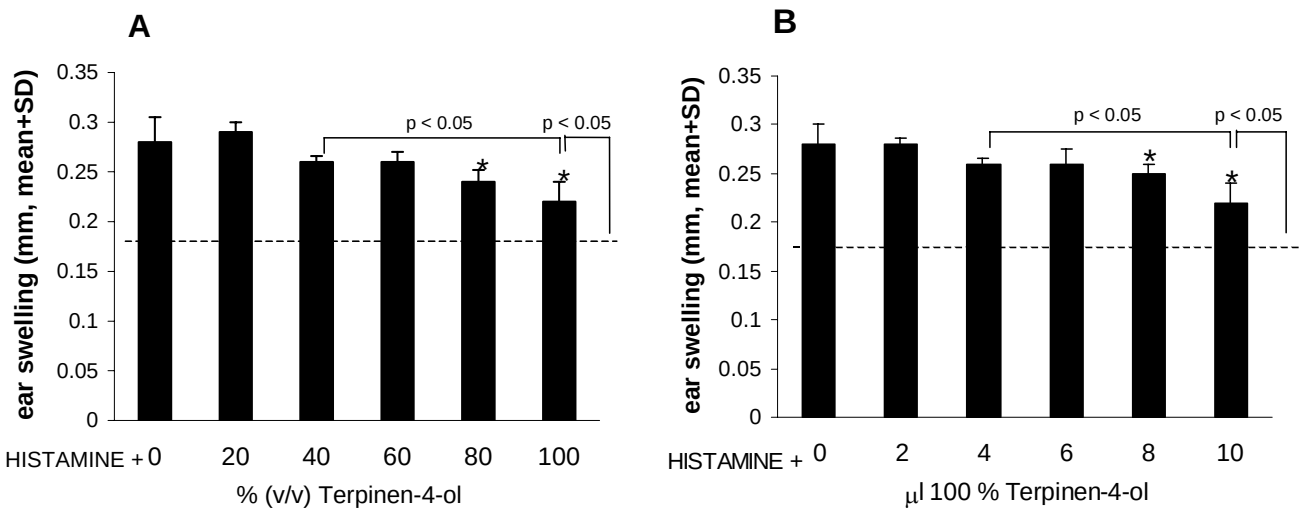

Figure 3.5. The effect of topical administration of terpinen-4-ol on the oedematous response induced by histamine injection in BALB/c ears. Ten µl of histamine diphosphate (60 mg/ml solution in 0.9% saline) was injected intradermally into the dorsal aspect of both ears of a mouse. Terpinen-4-ol was topically administered A. at increasing concentrations in a constant volume, or B. undiluted in increasing volumes to both sides of each mouse ear immediately after histamine injection. The data represent ear swelling (mm, mean+SD) 15 min after intradermal injection of 3 ears. An asterisk (*) indicates a significant suppression by TTO of histamine-induced swelling. The dotted line shows the extent of saline-induced swelling.

3.3 Discussion

By its effects on vascular endothelium and related permeability, histamine causes an oedematous response within minutes. The effect of TTO on histamine-induced oedema in ears from two strains of mice was examined. TTO applied immediately after, but not 30 min before intradermal injection of histamine, significantly suppressed oedema development. This study in murine ears complements a study that has recently been completed in our laboratory whereby TTO was topically administered to one inner arm of volunteers twenty min after histamine injection. The other arm was injected with histamine but no TTO applied. TTO significantly reduced the developing wheal while the flare that was maximal at the time of TTO administration was not affected by TTO administration (Koh *et al*, unpublished results). The study on human skin is similar to the results shown in the solid bars of Figures 3.2 and 3.3. This study in mice allows the inclusion of a control injection without histamine; in all mice, TTO augmented saline-induced swelling and highlights further the ability of TTO to reduce the oedema induced by histamine. Perhaps more importantly, this study in mice attempts to dissect the mechanism of control of oedema by TTO. In mice, there was no evidence for modulatory effects of TTO on nerves. This study also allowed the identification of terpinen-4-ol, the major water-soluble component of TTO, as responsible for oedema control by TTO. It remains unknown as to whether TTO reduced oedema formation or enhanced clearance.

We initially hypothesised that the effect of TTO alone, or with saline, may relate to an effect on mast cell function, with a greater effect hypothesised for the skin of C57BL/6 mice that have approximately double the number of mast cells in dorsal skin than found in BALB/c mice [34]. However, TTO had very similar effects in the two mouse strains. We therefore hypothesise that TTO is not functionally similar to lavender oil which has been shown to stablilise mast cells [35].

There is evidence that some cutaneous inflammatory reactions occur through release of neuropeptides such as substance P and calcitonin gene-regulated peptide from unmyelinated c-type sensory nerves. In addition, sensory neurons express histamine receptors [19]. By repeated administration of capsaicin to young mice, which causes the release of neuropeptides from small sensory cutaneous nerves, the peripheral sensory neurons of these mice were densensitized and unable to release neuropeptides upon subsequent stimulation. This model allowed the role of nerves and the interaction of TTO in histamine-induced ear swelling to be investigated. However, as seen with their non-capsaicin treated littermates, topical administration of TTO immediately after histamine injection significantly reduced histamine-induced ear swelling. Thus, TTO does not exert its anti-oedema effect by regulating the activity of peripheral sensory neurons.

3.4 Conclusion

The anti-microbial and anti-fungal properties of TTO are already well-established [1-3]. Anecdotal evidence suggests that TTO is useful for the relief of inflammatory skin conditions brought upon by insect bites or re-exposure to contact allergens such as plant chemicals or nickel. In the previous chapter we have shown that TTO can control the swelling associated with the efferent phase of a contact hypersensitivity response to a chemical hapten, a reaction that takes 24 h to develop and depends on the activity of immune lymphocytes . In this chapter, we demonstrate that TTO, in particular its main component terpinen-4-ol, may be effective in controlling histamine-induced skin swelling, a response that peaks after 20-30 min and which is often associated with immediate type allergic hypersensitivities.

Chapter 4. References

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