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Pine Oil Effects on Chemical and Thermal Injury in Mice and Cultured Mouse Dorsal Root Ganglion Neurons

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Abstract

A commercial resin-based pine oil derived from *Pinus palustris* and *Pinus elliottii* was the major focus of this investigation. Extracts of pine resins, needles and bark are folk medicines commonly used to treat skin ailments, including burns. The American Burn Association estimates that 500,000 people with burn injuries receive medical treatment each year; one-half of US burn victims are children, most with scald burns. This systematic study was initiated as follow-up to personal anecdotal evidence acquired over more than 10 years by MH Bhattacharyya regarding pine oil's efficacy for treating burns. The results demonstrate that pine oil counteracted dermal inflammation in both a mouse ear model of contact irritant-induced dermal inflammation and a 2nd degree scald burn to the mouse paw. Furthermore, pine oil significantly counteracted the tactile allodynia and soft tissue injury caused by the scald burn. In mouse dorsal root ganglion (DRG) neuronal cultures, pine oil added to the medium blocked ATP-activated, but not capsaicin-activated, pain pathways, demonstrating specificity. These results together support the hypothesis that a pine-oil-based treatment can be developed to provide effective in-home care for 2nd degree burns.

Keywords

scald burn; mouse; pine oil treatment; pain; edema; dorsal root ganglion (DRG) neuronal cells

INTRODUCTION

The aim of this research was to determine systematically whether effects of a 2nd degree burn (pain plus blister/edema/inflammation) could be prevented or reversed under rigorous and reproducible conditions in the laboratory. A commercial resin-based pine oil derived from *Pinus palustris* and *Pinus elliottii* was the major focus of this investigation. Extracts of pine bark, needles, and resins are folk medicines commonly used to treat skin ailments,

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including burns, as documented by reports from China, Japan, India, Russia, Kazakhstan, and Lapland (Pak et al. 2003, Simbirtsev et al. 2002, Sipponen et al. 2007 2008, Thornfeldt 2005). The investigation reported here was initiated as follow-up to personal anecdotal evidence acquired over more than 10 years by MH Bhattacharyya regarding pine oil's efficacy for treating burns; essentially, topical application of pine oil immediately after the burn event consistently prevented blister formation and, within minutes, eliminated the pain of 2nd degree burns that occurred in the home setting.

The American Burn Association estimates that 500,000 people with burn injuries receive medical treatment each year, with about 40,000 requiring hospitalization (Shields et al. 2007). One-half of US burn victims are children, most with scald burns. Pain is the common experience of all patients with burns regardless of cause, and the pain that they experience can be among the worst known (Connor-Ballard 2009). Persons in the US commonly seek relief of burns in the home setting by immersing the burn site in cold water to immediately relieve pain, then applying antibiotic ointment with a gauze wrap to retain moisture and prevent infection (Mayo Clinic 2012). With this widely-recommended approach, 2nd degree burns (pain with blister) can typically take several days to several weeks to heal, depending on their severity.

The results reported here demonstrate that pine oil counteracted dermal inflammation both in a classic mouse ear model of dermal inflammation induced by a contact irritant (12-O-tetradecanoylphorbol 13-acetate or TPA) and in a 2nd degree scald burn to the mouse paw. Furthermore, pine oil significantly decreased tactile sensitivity and soft tissue injury caused by the scald burn. Pine oil added to the medium blocked ATP-activated, but not capsaicin-activated, pain pathways in mouse dorsal root ganglion (DRG) neuronal cultures. These results support the hypothesis that a pine-oil-based treatment can be developed to provide effective in-home care for 2nd degree burns.

MATERIALS AND METHODS

Materials

Resin-based pine oil (PO) derived from *Pinus palustris* and *Pinus elliottii* was a commercial product obtained from T&R Chemicals, Inc. (Clint, TX). Several commercial pine oils were available from T&R Chemicals, each consisting of a non-alcohol and a terpene alcohol portion; the 60% pine oil product (pine oil with 60% terpene alcohol content) (composition shown in Table 1) was used for the studies reported here. The original pine oil used over many years by MH Bhattacharyya was a historical sample stored in a brown glass bottle with a ground-glass stopper; it was judged from its history to be a pine-resin-based oil (as opposed to bark extract, etc.). Trypsin, poly-L-lysine, capsaicin, resiniferatoxin, capsazepine and N-tert-butyl- α -phenylnitrone were obtained from Sigma-Aldrich (St. Louis, MO). Collagenase was purchased from Roche Applied Science (Indianapolis, IN). The fluorescent intracellular calcium release indicator fura-2AM was obtained from Molecular Probes (Eugene, OR). Thapsigargin used to test cytosolic calcium release at the end of each study was obtained from Calbiochem (La Jolla, CA).

Animals

For mouse ear edema studies, male ICR CD-1 outbred mice (25-30 g; 5-6 weeks of age) were obtained from Harlan Laboratories (Indianapolis, IN) and were allowed to acclimate for approximately one week prior to experimentation. For the paw burn studies, C57BL/6 male mice (19-21 g, Charles River) were fed standard chow and housed in a 25°C animal room with a daily 12hr light, 12hr dark cycle. For DRG cultures, C57BL/6 male mice (19 – 20 g, Jackson Lab) were fed standard chow and housed in a 24°C animal room with a reverse daily 12hr light, 12hr dark cycle. All experiments were approved by the appropriate IACUC committee at Georgia Health Sciences University or University of Kentucky/Lexington.

Pine Oil Effects on 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-Induced Inflammation of Mouse Ear

Four ICR CD-1 male mice (eight ears) were used per treatment group and three groups: vehicle-only control, TPA plus vehicle, and TPA plus pine oil. Mouse ears were treated with TPA according to the method of Fowler et al. (2003). At 2h and 4h after TPA, the ethanol vehicle or the original pine oil was applied as a 1:10 dilution in ethanol; each ear received 20 µl of the diluted pine oil per treatment time (2 µl PO/treatment). Ear thickness was measured using a caliper both before and at 18 hr after TPA treatment; special care was taken by an experienced investigator to avoid compression of the ear during this measurement. At 18 hr, a circular ear punch biopsy (4 mm²) was taken, weighed, and fixed in formalin. Histological evaluation included H&E staining and immunohistochemical staining for CD45 to quantify the number of infiltrating immune cells.

Pine Oil Effects on Tactile Sensitivity and Edema Following Scald Burn to Mouse Paw

Anesthesia—The C57BL/6 male mice undergoing paw burn were submitted to open-drop inhalation anesthesia in a bell jar using 30% v/v isoflurane in propylene glycol. The anesthetic solution, at 1.0cc per 500cc jar volume, was pipetted onto several cotton balls contained in beakers set below the bell jar platform on which the mice were placed. This approach separated the mice from the anesthetic solution, preventing irritation from direct physical contact with the isoflurane.

Burn Characterization—Based on mouse burn models described by Palmer et al. (2008) and Gao et al. (2009), three protocols were tested to produce a scald burn on the paw of C57BL/6 male mice, in preparation for evaluating effects of pine oil. The left hind paw was immersed for a measured time in water heated to a constant known temperature. The protocols tested were (water temperature/time): 65°C/5 sec; 65°C/2 sec; 60°C/2 sec. After burn, mice were allowed to recover to sternal recumbency on a warmed, bedding-free area before their return to individual housing.

Response Measurements—Tactile sensitivity was measured on each mouse using von Frey filaments to obtain tactile paw withdrawal threshold (PWT) values before the burn and at 30, 60, and 90 min afterwards (see below for detailed protocol). Just prior to each PWT measurement, thickness of the burned paw (measure of edema) was determined using a digital caliper, with measurements to the nearest 0.1 mm. The thickness of the unburned paw

of the same mouse was also measured at each time point to evaluate reproducibility of the measurement in a control paw. (Note that the burn protocol used for this measurement was mild enough that no systemic response, which might have swelled the opposite unburned paw, occurred). A single investigator, who was blinded to which treatment group each mouse belonged, obtained all caliper and PWT measurements. At 90 min after the burn, paws were fixed in 10% formalin, demineralized for one week with EDTA, and sections stained with H&E for evaluation of histological changes.

Pine Oil Treatment—Experimental mice were treated one time immediately after the scald burn with either the original pine oil used by MH Bhattacharyya or the commercial 60% pine oil. Although the original pine oil was tested, focus was placed on the commercial preparation because of its unlimited supply and the limited supply of the original oil. A 10 μ l aliquot of undiluted pine oil was applied to the burned paw, half on the dorsal and half on the ventral surface. Control mice were left untreated to characterize the progress of an undisturbed burn.

Paw Withdrawal Threshold (PWT) Test for Tactile Sensitivity—Tactile paw withdrawal thresholds (PWTs) were determined using standard von Frey filaments, ranging in force from 0.1 to 5g, following the adapted Dixon Up-Down method (Burnstock 1999; Palmer et al. 2008). The test apparatus consisted of a stand with a wire mesh platform and a clear glass bowl inverted on the mesh as a holding unit to contain the mouse (Figure 1). Before testing, each mouse was acclimated to the testing apparatus for 5 min or until it stopped exploring. Von Frey filaments were applied from below the wire mesh platform to the central region of the plantar surface of the mouse paw (Figure 1).

The following precautions were followed: filament was applied only when the mouse was stationary and standing on all four paws; for a valid withdrawal response, the tested hind paw was completely removed from the platform; if the animal walked immediately upon application, the filament was reapplied; flinching of tested paw was not a positive response, it must have been lifted from the platform. A single trial consisted of: application of a filament to the hind paw five times at ~5 sec intervals (or as soon as paw was appropriately placed on platform); if no withdrawal occurred in five applications, the next larger filament was used; withdrawal of the paw four or five times out of five applications was considered the threshold. This single threshold value was confirmed from two directions (Up-Down method): ascending in force, starting with filaments below the threshold value, until a full response is elicited; and descending in force, starting with filaments above the threshold value, until no response is elicited.

For the experiments reported here, the same tester conducted all PWT tests. The tester in all cases was blinded to the treatment group of each mouse.

Pine Oil Effects on Pain Transmission Neurons in Mouse Primary Dorsal Root Ganglion (DRG) Cultures

The ability of pine oil to inhibit activation of pain transmission neurons was tested in DRG primary cultures using live cell calcium imaging after activation with ATP and capsaicin (Chaban et al. 2003, Ma et al. 2009). Primary mouse DRG neurons were isolated, cultured

overnight (12-18 hr), and incubated with 5 μ M Fura-2 for 30 min (see protocol below). To test if ATP-activated pain pathways were modified by pine oil, neurons were treated with 10 μ M ATP (1 min) and then washed, 30 μ l 60% pine oil per ml medium (5 min), or a mixture of 10 μ M ATP plus 30 μ l 60% pine oil per ml medium (1 min) and then washed. An analogous test was conducted in which the effect of 60% pine oil on calcium mobilization was determined in DRG neurons activated by capsaicin (1 μ M, 30 sec) instead of ATP.

Intracellular Calcium Activity Measurements in Primary DRG Neuronal

Cultures—Primary mouse L4,5 DRG neurons were isolated from anesthetized mice and digested in fresh enzyme solution (10 mg trypsin+25 mg collagenase in 10 ml dissection solution) saturated with O₂ (Ma et al., 2009). After shaking in a warm water bath (speed 150 rpm, 35°C) for 1 hour, 4 ml of culture medium (MEM with FBS, antibiotics) were added to the flask to inactivate the enzymes. The cells were transferred into a 15 ml centrifuge tube and spun at 800 rpm for 5 minutes. The supernatant was discarded and the cell pellet re-suspended with culture medium. DRG cells were plated at a density of 4x10⁴/cm³ onto a poly-L-lysine pre-coated 12 mm glass-bottom tissue culture dish or 12 mm coverslips in 24 well culture plates (0.2 ml of cell suspension per well). Cultured DRG neurons were maintained in an incubator with 5% CO₂, 37°C overnight and all experiments performed on the subsequent day.

Cells were loaded with a dual excitation Ca²⁺ indicator dye, acetoxymethyl-ester Fura-2 (Fura-2AM, 5 μ M, Molecular Probes, Eugene OR), which was allowed to diffuse across the cell membrane in KREB's solution for 1 hour at room temperature in the dark, prior to imaging. Cells were then washed three times with KREB'S solution and the indicator was allowed to de-esterify for 30 minutes. During this process, the charged Ca²⁺ indicator is trapped inside the cell body, and any changes in the 340/380 ratio is an indication of intracellular Ca²⁺ mobilization. The coverslip with DRGs was mounted in a recording chamber containing 1ml KREB's solution attached to the stage of an inverted microscope at room temperature. Fluorescence studies were performed at room temperature using a Nikon TE-2000 inverted epi-fluorescence microscope with a 20X 0.9 quartz objective, and a cool snap HQ digital camera connected to a computer with Nikon Element program software (Nikon). Fura-2 loaded DRGs were excited alternately at wavelengths of 340nm and 380nm. The ratio of emitted light (340/380nm) detected at 510nm was used as an indirect ratiometric measurement of intracellular Ca²⁺ ([Ca²⁺]_i) levels.

Data Analysis

Descriptive statistics for data are expressed as mean $\pm$ SE, n. Paw withdrawal thresholds were evaluated with ANOVA, using Tukey's HSD post-hoc test where appropriate. Within subjects, comparisons were made for baseline and through time following treatment. Between subjects, comparisons were made at all individual testing time points. All statistical comparisons were evaluated at an alpha level of significance of 0.05.

RESULTS

Pine Oil Effects on TPA-Induced Inflammation in Mouse Ear

A study was conducted to test whether pine oil would counter the inflammatory response to TPA in the classic mouse ear edema model of inflammation. This study's relevance to burn injuries resides in the fact that inflammation is an integral part of the animal's response to burn (Ipaktchi et al. 2006, Schwacha et al. 2010).

As shown in Figure 2, treatment with the original pine oil at 2 hr and 4 hr after TPA caused a 3-fold decrease in the change in ear thickness (Figure 2A, top graph) and ear-punch weight (bottom graph) compared to the TPA-only group at 18 hr after TPA. The histological sections in Figure 2B show the clear reduction in ear edema due to pine oil treatment. In addition, infiltrating immune cell numbers were lower with pine oil treatment (19 ± 1 vs. 13 ± 3 CD45+ cells per field for TPA vs. TPA+PO, mean $\pm$ SE, $n=8$), although the decrease did not reach statistical significance.

Characterization of Scald Burn in Mouse Paw

This experiment was based on the projection that the paws of mice after a scald burn would show increased tactile sensitivity (measure of pain perception) and edema (measure of inflammation) and that these responses would depend on burn severity. The purpose was to identify a protocol that would generate a burn analogous to a 2nd degree burn.

As shown, all three burn protocols produced a striking increase in tactile sensitivity by 30 min after the burn in the no-treatment (NT) controls (Figure 3, left) (tactile PWT, 0.01-0.1 g at 30 min compared to 1.0 g pre-burn). By 90 min after the most severe burn (65°C/5 sec), the average paw was pink, swollen (70% increase in paw thickness) (Figure 3, right, blue bar) and significantly less sensitive to touch than after the other burn protocols (PWT, 0.4 g) (Figure 3, left, blue line). After 65°C/2 sec, the average paw was swollen at 90 min (50% increase in paw thickness) (Figure 3, right, red bar), showed marked edema in cross-section (Figure 4, middle-right section), and the PWT values remained extremely low through time (0.01 g) (Figure 3, left, red line). The mildest protocol (60°C/2 sec) produced the same prolonged sensitivity to touch (Figure 3, left, green line), with less edema at 90 min (Figure 3, right, green bar) and less histological damage (Figure 4, middle-left section). Increases in tactile sensitivity and paw thickness and changes in histology thus provided measures of the severity of responses to burn in the mouse paw.

Effects of Pine Oil on Scald Burn to Mouse Paw

65°C/2-Second Scald Burn—Both 60% PO-treated and no-treatment paws (NT controls) had become highly sensitive to touch 30 min post-burn, with a tactile PWT of ~0.01 g, compared to 1.0 g pre-burn (Figure 4A, left). By 90 min, 60% PO significantly decreased paw sensitivity, with a mean tactile PWT similar to the pre-burn paw (1.0 g). Note that 60% PO significantly suppressed the edema response to burn vs. NT control at 30 min (Figure 4A, right). However, at the later time points, paw thickness increases in 60% PO-treated paws were similar to those in the NT controls. Although not shown on the graph, the original PO produced similar results as did the 60%PO at the 90-min time point, with a

decrease in tactile sensitivity (PWT, 0.72 ± 0.17 g) (mean $\pm$ SE, n=5) and an increase in paw thickness ($80\% \pm 8\%$) (mean $\pm$ SE, n=5).

60°C/2-Second Scald Burn—At 30 min, both 60% PO-treated and untreated paws were highly sensitive to touch (Figure 4B, left). By 90 min, 60% PO significantly decreased tactile sensitivity compared to the untreated paw. Furthermore, after this milder burn, the NT control paw increased in thickness by 16% at 90 min, while 60% PO suppressed this edema response, as shown by a significant decrease in paw thickness at 90 min compared to NT controls (Figure 4B, right) ($p < 0.05$). Note that the changes in paw thickness with time for the no-burn control paws were near zero, demonstrating that the serial caliper measurements of paw thickness showed excellent replication, even with blinded groups. The PO effect was also shown by the near-normal histology in the treated paws at 90 min (Figure 4C, far right section vs. far left section). By both the tactile sensitivity and edema endpoints, a single early treatment with 60% PO reversed the responses to the scald burn.

Pine Oil Effects on Pain Pathways in Mouse Primary Dorsal Root Ganglion (DRG) Neuronal Cultures

The question was asked whether pine oil would block activation of primary afferent neurons responsible for transmitting pain responses in DRG neuronal cell cultures. Thus, we determined whether pine oil inhibited the cytosolic calcium response to ATP, a signaling molecule released from cells in response to burn injury and implicated in pain (Bleehen and Keele 1977, Zhao et al. 2008)

The upper trace of Figure 5 shows an example of the calcium response (340/380 ratiometric peak) to ATP treatment (1st blue arrow). While 60% pine oil alone gave no calcium response (3rd blue arrow), 60% pine oil totally prevented the response to ATP in the example shown (large white arrow). The calcium response to 10 μ M ATP returned at the end of the run (blue arrow), indicating that pine oil did not adversely affect cell viability. The lower bar graph illustrates the 35% average decrease in the ATP-induced calcium response after pine oil, considering all cells. The cell photomicrographs show the fluorescence increase in ~55% of DRG neurons after activation with ATP (arrows, lower right panel) and the decrease in that response after pine oil (arrows, upper right panel). Similar to ATP, capsaicin alone increased the DRG calcium 340/380 ratiometric peak (by 240%); however, 60% pine oil had no effect on calcium mobilized by capsaicin treatment (not shown). These results demonstrate that pine oil's decrease in the DRG response was specific to the purinergic class of pain pathway channels, and not due to general cytotoxicity or to TRPV pain pathway cation channel activation.

DISCUSSION

The *in vivo* results reported here demonstrate that pine oil significantly counteracted the edema response of the mouse to two different stimuli when the oil was applied topically shortly after initiation of the edema-causing event – TPA applied to the ear or scald burn to the paw. Pine oil also countered the striking tactile allodynia that resulted from the scald burn. These results expand on the personal experience of the senior author and on other reports of wound healing by products of pine resin (Forrest 1982, Pak et al. 2003, Rakhimov

et al. 2000, Mchedlidze et al. 1998, Simbirtsev et al. 2002, Sipponen et al. 2007, Sipponen et al. 2008). They provide a basis to investigate mechanisms by which pine oil improves the outcome of a burn event.

TPA-Treated Mouse Ear

Results with pine oil applied to the ears of mice after TPA treatment are consistent with inhibition of inflammatory processes. These are the first non-human *in vivo* data demonstrating this bioactivity of pine oil. Pine oil decreased the edema response to TPA by 61%, and the decrease was highly significant at 18 hr after TPA application (Figure 2); during this time, immune cell numbers in pine-oil-treated ears decreased to a lesser extent, by 30% (19 vs. 13 CD45+ cells/field), indicating a potential difference in timing of the two responses. deYoung et al. (1989) showed that the immune cell response to TPA in the mouse ear system lags considerably behind the edema response, with the edema peak at 6 hr and the immune cell peak at 24 hr after TPA. deYoung et al. (1989) also showed that the edema and immune cell responses differ among anti-inflammatory agents applied following TPA treatment, with some anti-inflammatory agents affecting both endpoints and some affecting only one. The greater decrease in edema than immune cells in response to pine oil is consistent with these results.

Scald Burn to Untreated Mouse Paw

In the untreated paw, edema arose in response to a scald burn, with the extent of paw swelling at 90 min decreasing with decreasing temperature and time of burn (Figure 3, right). For the paws with the most swelling (70% at 90 min), there was a *decrease* in tactile sensitivity at 90 min compared to 30 min, indicating that the burn alone may have caused sufficient damage to render the paws less responsive to the light touch of the von Frey filaments (Figure 3, left). The extensive swelling and decrease in tactile sensitivity indicate that the 65°C/5sec burn protocol was likely more severe than many in-home burns. More typical was the mildest burn, 60°C/2sec, which generated a reproducible 15% increase in paw thickness at 90 min, and maintained high tactile sensitivity through 90 min post-burn (low PWT) (Figure 3).

Palmer et al. (2008) created a mild thermal injury to the mouse hind paw by placing the hind paw of a mouse on the 56°C surface of a hot plate for 10 seconds. One ml of water on the hot plate surface facilitated conduction of heat, and a 10 g weight placed on the paw produced reproducible contact with the heated surface. As compelling as were the results of Palmer et al., we decided on the scald burn approach because the temperature was only crudely represented by the digital read-out of the hot plate, while the temperature of water could be exactly and reproducibly determined with a sensitive thermometer. In addition, the heat capacity of water is high enough that a given water temperature could readily be held constant over time with gentle warming. It is noteworthy that the striking tactile allodynia responses demonstrated in our mice after the two mildest scald burns were the same as those reported by Palmer after the surface paw burn – tactile PWT decreased from ~1g to ~0.1g at 30 min, with the latter decrease maintained through 90 min (Figure 4, left).

Gao et al. (2010) developed models of 1st and 2nd degree burns to the hind paw of the rat using a water-based scald burn. For the rat, a 1st degree scald burn was generated by 5 seconds at 70°C and a 2nd degree burn by 8 seconds at 70°C. Our testing began with a scald burn at 65°C for 5 seconds because of the smaller paw of the mouse. Exposure temperatures and times were decreased from there to produce a mild thermal injury (Figures 3 & 4) akin to that studied by Palmer et al. (2008).

Pine Oil Effects on Burn

In our study, tactile allodynia was counteracted by pine oil for both of the milder burn protocols (Figure 4). In addition, prevention of edema by pine oil, as evidenced by decreased paw swelling, was sustained through 90 minutes for the 60°C/2 sec burn and was transient for the 65°C/5sec burn, appearing only at 30 min. These results support the anti-inflammatory activity of pine oil demonstrated in the mouse TPA ear edema model (Figure 2). The protocol by which one investigator took all measurements and was blinded to each mouse's treatment group was important to the credibility of the sequential caliper measurements of paw thickness, especially for the group in which swelling was small after the mildest burn (15%). The fact that the measurements of paw thickness for the no-burn control paws were highly reproducible was also important (Figure 4) to validate the small burn-induced increases in this endpoint.

Pine Products for Wound Healing

Pine resin-based products for wound-healing are reported in historical documents as early as the 'Epitome' of Paulus Aegineta (~600-690 AD), in which pine resin was used to cleanse wounds (Forrest 1982). Applications of pine resin to wound healing have been passed on through the years as a folk medicine. More recently, 'Bialm' ointment, a pine resin preparation, has been tested for wound healing, including severe burns, by Pak et al. (2003) and Rakhimov et al. (2000) in Kazakhstan. For example, twice daily topical application of bialm ointment to third degree skin burn in rabbits decreased total healing time from 65 days to 26 days (Rakhimov et al. 2000). 'Biopin' ointment has been similarly studied in Russia (Mchedlidze et al. 1998, Simbirtsev et al. 2002). Sipponen et al. (2007, 2008) presented studies in which a pine resin preparation used by Lapp people of northern Finland was tested on refractory skin pressure ulcers, demonstrating that all ulcers healed within 6 months in 12 of the 13 patients (92%) in the resin group and in four of the nine patients (44%) in the control group ($P = 0.003$; power 73%). The results in this report thus add to a small base of studies that give credence to the proposal that pine resin contains components with valuable wound-healing properties. The property of most interest to this laboratory based on the experience of the first author – early and complete reversal of 2nd degree burn injury – is tested here for the first time.

Mediators of Pain and Inflammation in Mouse Dorsal Root Ganglion Cultures

Our results show that pine oil blocks activation of calcium channels by ATP but not capsaicin in DRG neuronal cultures, showing specificity of action. ATP activates P2X type receptors and voltage-gated Ca^{2+} channels (VGCC) known to be activated by algescic agents, while capsaicin activates TRPV channels, which are activated by noxious mechanical, heat, and chemical stimuli. Keratinocytes have been shown to release the nucleotide ATP in

response to mechanical and thermal stimuli as well as in response to frank injury (Burnstock 1999, Dussor et al. 2009). ATP binds to both the P2X ionotropic and P2Y metabotropic G-protein coupled family of receptors (Dussor et al. 2009, Burnstock 2007). The importance of nucleotide signaling in the intercellular communication among cells of the keratinocyte-axon complex after injury has been reported in recent studies (Mandidi et al. 2009, Molliver et al 2011). Our results show that pine oil does not interfere with DRG responses to the TRPV1 channel activator capsaicin (0.03-5 μ M), eliminating this ion channel as an involved player. However, pine oil clearly blocked DRG activation by ATP (Fig 5). With further study, the specific receptors activated by ATP that are inhibited by pine oil can be identified, to distinguish among P2X2, P2X3, P2Y1, P2Y2 and P2Y4.

Previous research has shown that TRPV4 channels can be activated by hypo-osmotic conditions occurring in edema, by lipid mediators released in injured tissue (arachidonic acid), and by IL-6, which has been shown to activate DRG neurons (Liedtke and Friedman 2003, Sommer and Kress 2004, Cao et al. 2009, Kochukov et al. 2009). The ability of pine oil to reduce DRG activation in response to the TRPV4 activators would provide additional insight into specific inflammatory pathways affected by pine oil.

Finally, Fowler et al. (2003) have shown that 22(OH)-cholesterol exerts its anti-inflammatory action by interaction with LXR, the hydroxy-steroid receptor the activation of which blocks TPA-induced inflammation in the mouse ear. Similar to this hydroxysteroid compound, multiple terpene alcohol isomers present in pine oil (Table 1) have a molecular asymmetry and a hydroxylated carbon atom attached to a mono-unsaturated 6-member hydrocarbon ring. Consequently, the pine oil alcohols may be close enough structural analogs of the hydroxy-steroids that they will also activate LXR, causing them to be similarly anti-inflammatory.

This initial study of resin-based pine oil clearly identifies a commercial pine oil preparation with burn mitigation properties worthy of subsequent investigation. Results document prevention of edema and early reversal of tactile allodynia following a scald burn in mice and demonstrate specific blockage of ATP-activated pain pathways in primary DRG neuron cultures. As with any study, the line of investigation followed to date has limitations. For example, a one-time application of undiluted pine oil was tested in the mouse paw burn model, with no evaluation of concentration-response or multiple oil applications. Furthermore, the control paws received no treatment; that is, they were not treated with an alternate oil, whose purpose would be to test the specificity of pine oil. Clearly, this study provides a basis for future studies regarding potential development of an in-home approach to treatment of 2nd degree burns

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Figure 1. Apparatus for Tactile Sensitivity Measurements

Mouse was acclimated to holding space on top of screen and underneath clear glass dome, which provided undistorted view of mouse. Von Frey filament attached to holder was pressed against plantar surface of mouse paw to determine whether mouse sensed presence of filament. Series of tests with filaments of increasing and then decreasing force were used to determine paw withdrawal threshold (PWT) (see text for detailed method).

Figure 2. Pine Oil Effects on TPA-Induced Changes in Mouse Ear

A) Effects on increases in ear thickness and ear weight, characteristic of TPA-induced ear edema. Bars show mean $\pm$ SE, $n=8$ /group; *** $p<0.001$ versus the control; ††† $p<0.001$ versus TPA alone (ANOVA + Student-Newman-Keuls). B) Effects on mouse ear histology. H&E, 20x. Mouse ear was treated with original pine oil or vehicle at 2 and 4 hr after TPA application. Mice were euthanized and punch biopsies from ears evaluated at 18 hr after TPA.

Figure 3. Effects of Different Scald Burn Protocols on Tactile PWT (Left) and Edema (Right) Responses of Mouse Paw

Left hind paw of no-treatment (NT) control mice were subjected to one of three indicated scald burn protocols, as described in text. Tactile PWT and paw thickness measurements were made in each mouse before and at 30, 60, and 90 min after the burn. Values are mean $\pm$ SE, 6-11 mice/group. Left: * = PWT significantly different from PWTs for other burn protocols at given time point. Right: Bars with same letter not significantly different. (ANOVA + Tukey-Kramer Multiple-Comparison test, $p < 0.05$).

Figure 4. Pine Oil Effects on Response of Mouse to Paw Scald Burn

A. Effects of 60% pine oil on tactile PWT (left) and edema (right) after 65°C, 2 sec scald burn to hind paw. Values are mean $\pm$ SE, 5-11 mice/group. * $p < 0.05$ vs. NT control at same time point (ANOVA + Tukey-Kramer Multiple Comparison). **B.** Effects of 60% pine oil on tactile PWT (left) and edema (right) after 60°C, 2 sec scald burn to hind paw. Values are mean $\pm$ SE, 5-11 mice/group. * $p < 0.05$ vs. NT control at same time point (ANOVA + Tukey-Kramer Multiple Comparison). **C.** Cross-sections of mouse hind paw for no burn control (left, normal tissue), no treatment (NT) controls 90 min after scald burn at 60°C, 2 sec (left middle) and 65°C, 2 sec (right middle), and PO-treated paw 90 min after 60°C, 2 sec scald burn (right) (H&E, 4x).

Figure 5. Pine Oil Effects on Pain Pathways in Mouse Primary DRG Neuronal Cultures

Cells were isolated and cultured as described in text. Top graph shows calcium response (340/380 ratiometric peak) to ATP treatment (1st blue arrow) and lack of response to ATP in presence of 60% pine oil (white arrow). Bottom left graph shows mean decrease in ATP-induced calcium signal by pine oil for all cells evaluated (n = 20 cells). Bottom right panel illustrates fluorescent calcium signal induced by ATP (bottom) and blocked by pine oil (top).

Table 1Chemical Composition of 60% Pine Oil^a

Component	% (v/v)
TERPENE HYDROCARBONS	
α-Pinene	0.5
Camphene	0.2
β-Pinene	0.1
Limonene	2.9
γ-Terpinene	3.5
p-Cymene	2.4
p-Menthadiene	2.2
Terpinolene	28.4
Total Terpene Hydrocarbons	40.2
TERPENE ALCOHOLS	
Secondary Alcohols	0.4
α-Fenchol	13.8
Terpinene-4-ol	2.7
cis-beta-Terpineol	2.4
Isoborneol	1.6
α-Terpineol	38.9
Total Terpene Alcohols	59.9
Grand Total	100.0

^aComposition determined by GC, provided by T&R Chemicals, Inc., Clint, TX (personal communication).