Experimental pain models for the evaluation of next-generation analgesics in clinical pharmacology studies

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CHAPTER 8

Investigation of the sensitizing properties of a topical ethanolic 1% capsaicin formulation, and its applicability in a nociceptive test battery

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ABSTRACT

OBJECTIVE In experimental context, capsaicin is used as a model to temporarily induce cutaneous sensitization to heat and mechanical stimuli, and alter skin properties. These effects, however, vary between capsaicin formulations. We investigated whether an ethanolic 1% capsaicin formulation could induce sensitization, and whether it interfered with other pain tests, to determine if it could be included in a multi-modal test battery and used in early-phase analgesic drug studies.

METHODS This was a two-period open-label study in ten healthy male volunteers. Sensitization induced by ethanolic 1% capsaicin was evaluated by determining pain thresholds using a heat pain test, Von Frey test, laser evoked potentials, and in combination with an validated evoked pain test battery (pressure-, cold pressor, electrical burst and electrical stair pain tests). Skin redness, erythema and blood perfusion were evaluated using multispectral- and laser speckle contrast imaging. Data were analyzed with a repeated-measures ANCOVA.

RESULTS Ethanolic 1% capsaicin induced a mean peak pain of 4.4 (on an 11-point scale, t = 0.5 h), significantly induced primary heat sensitization (Estimate of Difference (ED) primary vs untreated area: -9.8%, p<.0001) and secondary mechanical allodynia (response in time different from '0': p<.05). The secondary allodynic response was more evident in Period 1 compared to Period 2, but did not significantly differ (p=.211). Capsaicin also increased skin redness (ED: 0.11 Arbitrary Units (AU), p<.0001) and blood perfusion (ED: 44 AU, p<.0001) compared to untreated skin, and did not clearly interfere with other pain models.

CONCLUSIONS The ethanolic 1% capsaicin solution induced stable primary sensitization, skin redness, was well-tolerated and did not evidently interfere with other tests. Secondary allodynia was induced significantly, yet variable. The ethanolic 1% capsaicin solution is suitable as model for use in early-phase drug studies in the context of a multi-modal nociceptive test battery, but leaves room for further improvement.

INTRODUCTION

Capsaicin, the active component in chili peppers, is a chemical irritant often used in clinical setting. Capsaicin induces burning and painful sensations through highly selective interaction with transient receptor potential cation channel subfamily V member 1 (TRPV1), present on C-fibers and a subset of Aδ-fibers. [1–3] Conversely, prolonged exposure to high concentrations (e.g., 8%) leads to analgesia lasting for months by reducing TRPV1-expressing nociceptive nerve endings. [4] TRPV1 can be activated by noxious heat (≥ 43 °C) and physical abrasion, allowing capsaicin in lower concentrations (≤ 3%) to be used as a challenge agent to induce primary sensitization to heat and mechanical stimuli by modulating peripheral afferent nerves at the treated site. [5,6] Capsaicin also exerts effects in the central nervous system (CNS) by sensitizing nociceptive neurons to their normal or subthreshold afferent input (i.e., central sensitization), which is hypothesized to be due to transiently increased neuronal excitability in the dorsal horn. [7,8] Capsaicin-induced central sensitization may, for example, be evaluated by quantifying the mechanical allodynic response in the area surrounding the site where the capsaicin was administered (i.e., secondary allodynia).

In experimental settings, capsaicin is mostly either topically administered as a cream or ethanolic solution, or injected intradermally. [6,7,9–14] While scientific publications about the effects of capsaicin on primary sensitization and neurogenic inflammation (e.g., erythema and vasodilation) mostly agree, reports on capsaicin-induced secondary allodynia do not: only half of the studies demonstrate secondary effects of topical capsaicin formulations. [15] In a previous human experimental pain study, we could only demonstrate primary, but not secondary effects of a 1% capsaicin cream formulation. [16]

For a topical drug to be efficacious, sufficient skin penetration of the active ingredient and skin permeation are key. However, skin penetration is significantly influenced by a drug's physicochemical properties – and therefore challenging to optimize. [17] The cream formulation we previously tested may have inadequately penetrated the skin, thereby limiting induction of secondary allodynia. Chemical penetration enhancers including ethanol can increase (topical) drug flux and skin permeation, which may lead to higher efficacy of the active ingredient. [18–20] An ethanolic capsaicin solution formulation may therefore be superior in

inducing secondary allodynia, as suggested by other studies with such a formulation. [6,9,13,21,22]

For an experimental pain model to be of use for evaluating (novel) analgesics in the context of a multimodal pain test battery, it may not influence other measurements and the results should be reproducible. [16] A proof-of-concept trial which validates the use of a new model in context of existing methods is therefore warranted. In our clinical unit, we use a validated multi-modal test battery with distinct tests that do not interfere with one another, and allow for profiling and benchmarking of drugs against each other. [23]

Here, we evaluated whether a topical ethanolic 1% capsaicin solution could induce primary and secondary sensitization without influencing other tests, to serve as an extension to our nociceptive test battery.

METHODS

General considerations

The study was conducted at the Centre for Human Drug Research (CHDR, Leiden, The Netherlands), in accordance with the Declaration of Helsinki of 1975, its amendments and the Guideline for Good Clinical Practice. This study was registered in the Netherlands Trial Register under No. 7704, ToetsingOnline No. NL68698.056. and approved by the Medical Ethics Committee Stichting Beoordeling Ethiek Biomedisch Onderzoek (Stichting BEBO, Assen, The Netherlands) before any assessments took place.

Study design

This was a two-period, open-label, proof-of-concept study in 10 healthy males. Enrolled subjects attended the clinic on two identical visits lasting one full day each, with a wash-out of at least 7 days in-between. A telephonic follow-up 5-9 days after the last capsaicin administration (i.e., last dose) concluded study participation.

Screening was planned up to 42 days before the first study day. Written informed consent was voluntarily provided by all subjects prior to any assessments taking place. Male volunteers, aged 18-45 (inclusive), with a BMI between 18-30 kg/m2 (inclusive) and that were overtly healthy as

confirmed by a comprehensive medical evaluation including vital signs, medical history review and previous or chronic pain symptoms, were eligible to participate. Subjects that reported to have significant allergic reactions (urticaria or anaphylaxis) to capsaicin, or with a dark skin type (Fitzpatrick V and VI), widespread acne, tattoos or scarring on the volar forearms were excluded.

The 1% capsaicin solution (see next section) was applied at screening to make subjects familiar with the sensation and exclude those allergic to the solution or reporting to have intolerable pain after administration. In addition, a training session for all pain tests except the von Frey assessment was part of the screening assessments to familiarize subjects. Those indicating to be intolerable or too tolerant were excluded. Subjects were found to be too tolerant when achieving tolerance at >80% of maximum input intensity for the pressure, electrical or cold pain test The training also included determination of the individualized laser stimulus threshold for the LEP assessment (test procedures details in section **Study procedures**).

Study drug

A 60% ethanolic, topical 1% capsaicin solution was used and produced under Good Manufacturing Practice (GMP) conditions at Tiofarma BV, Oud-Beijerland, The Netherlands. The solution contained capsicum oleoresin US Pharmacopeia (USP), ethanol 96% pure and purified water.

At screening and in the morning of both study days, 50uL (= 0.5 mg) of the 1% capsaicin ethanolic solution was applied topically on a predefined 3×3 cm area on the dominant volar forearm (i.e., primary area), after which it was occluded for 30 minutes. Household film was used for occlusion for the first two subjects on the first study period, but pressed the capsaicin outside of the intended area of application and therefore was replaced by Tegaderm film of 6x7 cm (3M, USA) for all subsequent study days. A 3×3 cm silicone mall placed prevented the solution from spreading outside the intended area of application. Remaining solution was carefully wiped off the skin towards the middle of the 3×3 cm area after 30 minutes.

Adverse events (AEs) were recorded to confirm safeness of using the ethanolic 1% capsaicin formulation.

Study procedures

EVOKED PAIN TEST BATTERY At screening and during each visit, a validated battery of pain tests was performed (**Figure 1**). All pain tests were equipped with a maximum safety cut-off to minimize the risk of harming subjects. [24]

The pain test battery was performed as previously described. [25,26] In summary, subjects were assigned to a separate room that did not have any form of distraction and seated comfortably in a chair. For each test but the heat pain test, subjects were asked to hold a hand-held electronic visual analogue scale (eVAS), with which they could indicate their currently perceived pain intensity. The eVAS ranged from 0 up to 100. 0 was defined as 'no pain', sliding > 0 defined the Pain Detection Threshold (PDT), and 100 defined the Pain Tolerance Threshold (PTT; 'worst pain tolerable').

For the heat pain tests on capsaicin-treated and untreated skin, a 3×3cm thermode (QSense, Medoc, Israel) was placed first on the area where capsaicin was applied (i.e., primary/capsaicin-treated area). The thermode gradually increased with 0.5°C/s starting from 32°C. Subjects were given a hand-held feedback control and asked to click the button on the control when the heat stimulus was first perceived as painful (PDT). As a safety precaution, no heat PTT was measured and 50°C was used as cut-off temperature. This procedure was repeated next on the non-dominant arm at an area contralateral to that of where capsaicin was (planned to be) applied (i.e., control/untreated area). Per timepoint and per area, the average of triplicate measurements was used for further analysis.

The short-form McGill Pain Questionnaire (SF-MPQ, Dutch version) was used to evaluate the affective and sensory components of the pain perceived following the heat-, pressure-, electrical burst-, electrical stair- and cold pressor pain task. [27,28]

LASER EVOKED POTENTIAL (LEP) ASSESSMENT To assess changes in cortical brain response, LEPs were recorded following laser stimulation (LS) in a quiet room with minimal illumination using adequate safety precautions. A laser (Nd:Yap, Stimul 1340, Electronic Engineering) generated stimuli with a 5 mm diameter, 5 msec duration, random inter-stimulus intervals of 6-8 s and individual stimulus strength as defined at screening (see next paragraph). 20 stimuli were administered per location, during study days first on the secondary area (dominant arm, **Figure 2**), then control area (non-dominant arm) and ending with the

primary area (dominant arm). The stimulation site was moved slightly within the defined area after each stimulus to avoid skin damage and nociceptor sensitization/habituation. [21,29,30] After each set of 20 stimulations, the subject reported peak pain using an 11-point NRS for each specific area. Cortical responses to LS were recorded using an EEG system (REFA32, Twente Medical Systems international (TMSi), Oldenzaal, the Netherlands) and collected with a 10-20 cap system. To minimize recording artefacts, subjects were instructed to keep their eyes open, focus and stay relaxed. Subjects were asked to push a hand-held reaction button when a stimulus was felt. The amplitude (μV) and latency (msec) of the maximum negative peak between 150 and 300 msec (N2), the maximum positive peak between 250 and 360 msec (P2) and N2P2 peaks that were observed in each EEG were used for analysis.

As part of the screening procedures, the individual threshold of each subject was determined using a validated script, following related literature. [22] Briefly, the laser stimulus was pointed at non-treated skin on the non-dominant volar forearm. The stimulus increased from 0 to a maximum of 2.0 J, with increments of 0.25 J/step. Subjects reported if the stimulation was perceived as a sharp pinprick by answering a concise yes/no question following each step. If the stimulus was felt as a sharp pinprick or 2.0 J was reached, the ramp was repeated for a total of three times. The average result was multiplied by 1.5 and, if needed, rounded off to a lower value for safety purposes. That threshold was used for that specific subject throughout the remainder of the study.

SECONDARY MECHANICAL ALLODYNIA (VON FREY TEST) At the start of each study visit, eight spokes that divided a circle equally were drawn on the volar forearm to quantify the area of secondary allodynia (**Figure 2**). Pre-capsaicin application, individualized perception to mechanical pain was determined using Von Frey filaments (OptiHair, MRC systems GmbH, Heidelberg, Germany) with strengths of 128, 256, 362 and 512 mN. The strength preceding the one the subject reported as being just painful at first was used to determine allodynia for that subject.

After capsaicin administration, the assessment started on the north spoke at the most outer point from the center of the primary area and moved to the middle with steps of 5 mm. Once sensation changed from nearly painful to painful, that point was determined to be the border of the allodynic area. This assessment was repeated for all spokes in a clockwise fashion. The allodynic area was quantified in mm^2 using individual

values for each spoke. A 5 mm border zone for each spoke surrounding the primary area (i.e., the 3×3cm capsaicin application area) was used to minimize the risk of reporting false positive effects.

SKIN ANALYSIS – ERYTHEMA AND BLOOD FLOW ASSESSMENTS
The assessments were performed in a temperature-controlled area (approximately 22°C) where subjects were accommodated to the temperature for at least 15 min. Procedures employed to assess effects of capsaicin on the skin using multispectral and laser speckle contrast imaging, have been described extensively described elsewhere. [31–33]

In brief, capsaicin-induced erythema (defined here as the CIELAB colour space a* value) and redness (defined as the haemoglobin average level [34]) were measured using a multispectral imaging device (Antera 3D, Miravex, Dublin, Ireland). The CIELAB a* value is a colorimetric score of redness based on the harmonized CIELAB color space, whereas the haemoglobin score is based on an algorithm of the Antera 3D device that assesses skin redness. The regions of interest for both assessments were size matched at the site of application, and at the untreated site used as control (i.e., same area of skin on the dominant and non-dominant arm, respectively). Skin blood perfusion (i.e., basal blood flow) was quantified using Laser Speckle Contrast imaging (LSCI; PeriCam PSI System, Perimed AB, Järfälla, Sweden). [31]

Statistical considerations and analysis

Analysis was performed using SAS for Windows version 9.4 (SAS Institute Inc., Cary, NC, USA). No adjustments for multiple comparisons were employed as the study was explorative.

For the Von Frey test, secondary mechanical allodynia was defined as a response > 0mm^2 outside the primary and border area. A responder was defined as reporting to have an area of secondary mechanical allodynia > 0mm^2 in both study periods. Intra-subject variability was visualized by calculating the mean difference of the allodynic response per subject (i.e. Period 1-Period 2). Von Frey test results for the first study period of the first two subjects were excluded from analysis as the solution accidently spread outside the primary area, and therefore was deemed to preclude proper assessment of effects on the secondary area (also see **Figure 2**.)
Repeatedly measured pharmacodynamic data were analyzed with a

mixed model analysis of covariance with group, area (if applicable: control, primary or secondary area), time, visit, and interaction effects as fixed factors and subject, subject by area and subject by time as random factors and the (average) baseline measurement as covariate. Contrasts for primary versus (vs) control, and if applicable secondary vs control, primary vs secondary and secondary mechanical allodynia vs '0' were calculated within the model.

For each endpoint, estimates of the difference (ED) were generated for specified contrasts, and back transformed EDs in percentage for log transformed parameters. In addition, 95% confidence intervals (95% CI; in % for log-transformed parameters) and Least Square Means (LSMean) (the geometric means for log transformed parameters), and the p-value for each applicable contrast was reported.

RESULTS

Demographics and safety

A summary of subject characteristics is included in **Table 1**. 10 male subjects were enrolled as planned and completed the study.

Seven subjects reported a total of 16 AEs, of which eight were related to LS study procedures and four were reported as a mild burning sensation on the capsaicin application site. Other AEs were unrelated to study conduct (e.g., nasopharyngitis). One AE was moderate in severity (second degree burn after laser stimulation), all others were mild.

Pain test results

EVOKED PAIN TEST BATTERY Results are summarized in **Table 2**. Capsaicin significantly lowered heat PDTs on treated skin compared to heat PDTs on untreated skin (ED: -9.8%; 95% CI: -10.8 – -8.6%; p<0.001), which peaked around 2 h post-administration and lasted until the last measured timepoint (10 h post-administration) (**Figure 3**). Effects were similar in both periods. The affective and sensory perception of heat pain were both significantly increased as noted on the SF-MPQ (affective, ED: 0.07; 95% CI: 0.01 – 0.13; p <.05; sensory, ED: .16, 95% CI: 0.11 – 0.20; p <.001).

No significant differences over-time, or period effects (period 2 vs period 1) were noted for PDT or PTT for the other evoked pain models included

in the test battery (i.e., cold pressor-, electrical burst-, electrical stair-, pressure pain test and CPM paradigm) (**Table 2** and **3**). No effects were observed on the SF-MPQ endpoints for the cold pressor-, electrical burst-, electrical stair- and pressure pain test.

SUBJECTIVE PAIN RATING AND VON FREY TEST Subjective capsaicin pain ratings using a NRS were transiently and significantly increased after application, peaking shortly after administration (t = 0.5 h) to an estimated mean of 4.4 (on 11-point scale; effect over time p <.001). Pain ratings were comparable in both periods (estimated mean Period 1: 1.6, Period 2: 1.5; p=0.368) (**Figure 3**; **Table 2**).

Capsaicin significantly induced secondary mechanical allodynia (response for n=10 subjects over time versus '0' (i.e., no secondary allodynic response): estimated mean period 1: 728.7 mm^2, estimated mean period 2: 497.8 mm^2; p<.05). (**Figure 3**; **Table 2**). Only a subset of subjects (n=6, of n=8 that could be included in the analysis (see section **Statistical considerations and analysis**) reported to have allodynia in both periods.

Although no significant difference between periods was observed (period 2 versus period 1: ED: -230.8 mm^2; 95% CI: -717.6 – 255.9 mm^2; p>.2), effects were consistently more pronounced in period 1 than period 2, with the exception of the 30 min time point (**Figure 4**; **Table 2**).

LEP TEST Pain response to LS as measured with a NRS significantly differed between the primary and control area (ED: 0.76, 95% CI: 0.46 – 1.06, p<.0001), and between the secondary and control area (ED: 0.57, 95% CI: 0.27 – 0.87, p<.001). (**Table 2**).

No significant effects of capsaicin were noted for the response time to LS, or peak-to-peak amplitude for the N2-P2 peaks (**Table 2**).

Skin analysis

Compared to the untreated area, capsaicin significantly increased skin blood perfusion on the treated area (ED: 44.02 arbitrary units (AU); 95% CI: 39.20 – 48.84; p<.001) and significantly induced skin redness (haemoglobin levels for treated vs untreated skin: ED: 0.11 AU; 95% CI: 0.07 – 0.15 AU, p <.001). No significant erythemic effect was observed (CIELAB a* score treated vs untreated skin, ED: .37 AU, 95% CI: -0.08 – 0.82; p>.1) (**Figure 3**, **Table 2**).

DISCUSSION

We evaluated the sensitizing effects of an 1% capsaicin ethanolic solution, when incorporated as model in a validated evoked pain test battery. Results indicate that this formulation induced significant and tolerable primary heat sensitization without evidently influencing other pain tests. Secondary mechanical allodynia was also significantly induced, yet variable and only observed in a subset of subjects.

Various capsaicin formulations and administration routes are used in experimental context to induce sensitization, each with its own strengths and weaknesses. Intradermal (ID) injection, for example, can elicit reproducible and long-lasting secondary allodynia without requiring other possibly influencing factors as a heat sensitization procedure (i.e., kindling with a heat thermode), a tactic commonly employed for topical formulations to stabilize and increase duration of capsaicin effects. [35] While 80-100% of subjects receiving an ID capsaicin injection report to have secondary allodynia, [35] we deliberately decided not to test an ID formulation. The procedure is more difficult and invasive compared to topical application, but also induces a nearly maximal pain sensation (NRS of ~9 out of 10), [36] which likely will result in significant subject drop-out rates in early-phase drug studies with a multi-period cross-over design that require (highly painful) injections in every study period. We develop the capsaicin model for such designs specifically, therefore believe an ID injection is not suitable. The limited increase in NRS (a maximum LSM of 4.4) confirms that the ethanolic formulation is tolerable and applicable for its intended use (**Figure 3**).

Rather, hoping to increase the secondary allodynic response, we opted to change our formulation to one with enhanced skin penetrability by switching from cream to an ethanolic solution at a concentration (i.e., 60% ethanol) found stable by the manufacturer. Primary sensitization to heat was evidently more induced by the ethanolic- compared to the cream formulation (ED of Heat PDT vs control ethanolic formulation: -9.8%; ED of Heat PDT vs control cream formulation -3.85%), and secondary allodynia was repeatably induced by the ethanolic formulation whereas the cream formulation could not produce any notable effects. [16] These results suggest that between the formulations we tested, the ethanolic option indeed is superior in inducing secondary sensitization.

Nonetheless, also when using the ethanolic solution formulation, only a subset (n = 6) of subjects reported to have secondary mechanical allodynia in both study periods and the allodynic area was limited (**Figure 3**). It is not likely that a higher concentration would have yielded more pronounced effects, as a recent review found no correlation between topical capsaicin concentrations and allodynic area. [35] Conversely, a correlation was found between heat-kindling of the treated area, and enhancement of the allodynic response. [35] We did not employ that procedure here, as we did not observe any differentiating effects of kindling in the previous capsaicin cream study. [16] While publicly available literature only sparsely discusses the duration of the allodynic response, the kindling interval in our previous study with capsaicin cream (every ~2 hours) was not in line with other studies that successfully employed the procedure (every 45 min). [10,16,37,38] It is suggested to re-evaluate whether kindling may be used to further optimize the capsaicin model.

We found no significant difference in the secondary allodynic response between both periods (ED: -230.8 mm2; p>.2), although it should be noted that effects in period 1 were evidently more pronounced at all timepoints except for 30 min (**Figure 4**). Decreased sensitization of TRPV1 is known from e.g. high-dose (8%) topical capsaicin administrations, [4] but in experimental study-context a decreased response after repeated capsaicin administration is only described once elsewhere. [39] Cavallone et al. reported decreased secondary hyperalgesia to Von Frey testing – but not for allodynia using brush strokes – after second capsaicin administration. They determined this was in contrast to others as well. [39] Further studies with our ethanolic 1% capsaicin formulation are warranted to confirm whether the decreased response was a chance finding due to small sample size, or rather due to habituation effects.

As an alternative to the use of capsaicin to induce secondary sensitization, other models may be considered, such as high-frequency electrical stimulation (HFS). This relatively novel method selectively induces secondary allodynia at an amplitude similar to ID capsaicin and lasts for several hours. [40,41] Unpleasantness of the procedure is reported to be meagerly lower than ID capsaicin injection, so it has to be evaluated if that will preclude its use in multi-period crossover drug studies. [42,43]

We found no significant influence of the 1% capsaicin ethanolic formulation on any of the other nociceptive tests (**Figure 3**, **Table 2**). The (LSMean) pain thresholds over the day were comparable to the previous

capsaicin study, when taking into consideration that the data of the other study is of a larger sample (n=18) and placebo treatment arm of a cross-over study, where this was an open-label proof-of-concept study in n=10 subjects without treatment (**Table 3**). In this study, we did note an unexpected slight decrease over time of heat PDTs on untreated skin that was comparable in both periods (**Figure 3**). There was no such trend on untreated skin in the proof-of-concept capsaicin cream formulation study. [16] We could not find a clear reason as to why in this study heat PDTs on untreated skin were decreased. We did change the assessment order (i.e., for heat PDT first evaluate the treated area before the untreated area, where it was performed vice versa in the capsaicin cream study), and switched to an air-cooled thermode (QSense 3×3 cm thermode, Medoc) from a water-cooled one (TSAII 3×3 cm thermode, Medoc, Israel). We do not believe that the change in order or change of capsaicin formulation is causative, as the heat tests were performed on two distinct extremities (i.e., dominant and non-dominant arm). We cannot rule out that the change of equipment is causative, as heat PDTs on untreated skin were also slightly decreased in two other studies with the same capsaicin cream that evaluated heat PDTs using the air-cooled QSense rather than water-cooled TSAII device. [26,44] We do believe it is unlikely as we followed the same test procedures and used a thermode of equal size (3×3 cm – studies suggest that mainly the contact area may affect the allodynic response). [39,45] Nevertheless, these observations do not limit the use of the ethanolic formulation in early-phase drug studies, as long as designed properly. In a placebo-controlled cross-over design, subjects are balanced per calculated contrast. This ensures that such variability, if any, is present evenly in every period and thus effectively is cancelled-out when calculating analgesic drug effects compared to placebo response. [46]

One other key characteristic of capsaicin is that it transiently increases skin redness and induces erythema. [47,48] Haemoglobin average levels, a surrogate for skin redness, and blood perfusion were significantly increased by capsaicin as expected (both p<.0001; **Table 2**). While evidently increased shortly after capsaicin administration, we found no significant effect on the erythemic response (evaluated with CIELAB a* score) (**Table 2**). Sparse evaluation of this endpoint due to the many other tests performed on a single day, in combination with black marker drawings on the skin (used for quantification of the allodynic response (**Figure 2**) may have hindered proper evaluation of CIELAB a* values. This confounding

effect was not noted on the other skin analysis endpoints as the haemoglobin score is based on an algorithm rather than colorimetric scoring with the CIELAB a* results, [34] and because skin blood low was measured using laser speckle imaging rather than colour spectrum-based imaging.

The results presented here are to be read with the following considerations. Pain perception and the contribution of TRPV1 to nociceptor excitability – through interaction with gonadal hormones –significantly differs between men and women. [49,50] To optimize our chance to find significant effects in this exploratory study, we only included men – which limits the conclusions drawn to that population. The temperature ramp setting used for determining heat PDTs was accidently set too low (0.1 °C/s instead of 0.5 °C/s) during data collection of both periods for one subject, and of one period for two subjects. As a conservative approach, the data presented here are without those results, while noting that this error did not affect the effects noted (ED heat PDT capsaicin- vs untreated skin, without incorrect measurements: -9.8%, same ED with all measurements included: -10%). Lastly, the instructions given to subjects for reporting of secondary mechanical allodynia may have been suboptimal (i.e., when a change from ''nearly painful to painful'' was perceived). While a Von Frey strength was determined pre-dose for which the subjects confirmed to not feel pain (see section **Secondary mechanical allodynia (von Frey test)**), we cannot rule out that 1) this perception changed over-time, and 2) that the allodynia was perceived similarly across the whole area where it was quantified (**Figure 2**). For future studies, it may be less biased and more in line with procedures of other investigators to 1) perform a baseline measurement (i.e. pre-capsaicin) identical to how the test is performed at subsequent timepoints, and 2) ask subjects for the distinct detection of an increase in perception to the mechanical stimulus (i.e. *'distinct change in sensation as increased burning, tenderness, more intense pricking, or an unpleasant sensation'*). [38,39]

In conclusion, the 1% capsaicin ethanolic formulation induced primary heat sensitization, secondary mechanical allodynia, and increased skin blood flow and erythema. The pain induced by the capsaicin application was tolerable, without evidently interfering with other measurements. The model therefore is suitable for use in early-phase drug studies as extension of a multi-modal nociceptive test battery, but leaves room for further improvement.

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Table 1 Summary of Demographic characteristics.

Demographic category	Number (N = 10)
SEX, N (%)	
Male	10 (100%)
AGE (YEARS)	
Mean (SD)	23 (3.7)
WEIGHT (KG)	
Mean (SD)	77.3 (7.8)
HEIGHT (CM)	
Mean (SD)	181.7 (5.3)
BMI (KG/M^2)	
Mean (SD)	23.4 (1.5)
FITZPATRICK SKIN TYPE	
Type II	4 (40%)
Type III	4 (40%)
Type IV	2 (20%)

m: centimeters, kilograms, m^2: square meters, SD: standard deviation.

Table 2 Summary of statistical analysis.

Endpoint	Contrast	1st LSM[1]	2nd LSM[1]	ED	95% CI Lower	95% CI Upper	p-value
NRS CAPSAICIN PAIN							
	Time (response-'0')						**<.0001**
	Period 2-Period 1	1.5	1.6	-0.2	-0.6	0.2	.37
	Von Frey Time (response-'0')						**<.05**
	Period 2-Period 1	299.4 mm^2	492.3 mm^2	-193 mm^2	-521.5 mm^2	135.5 mm^2	.21
HEAT PDT							
	Primary-control	36.1 °C	40 °C	-9.8%	-10.8%	-8.6%	**<.0001**
	Period	-	-		-	-	0.63
	MPQ: affective (primary-control)	.38	.31	.07	.01	.13	**.02**
	MPQ: sensory (primary-control)	.70	.55	.16	.11	.2	**<.0001**
LS							
Reaction time	Primary-control	724.3 ms	720.8 ms	3.5 ms	-29 ms	35.9 ms	.83
	Secondary-control	715.5 ms	720.8 ms	-5.3 ms	-38.5 ms	27.9 ms	.75
Amplitude N2-P2 *peaks*	Primary-control	23.9 uV	24.1 uV	-0.2 uV	-2 uV	1.5 uV	.79
	Secondary-control	23.1 uV	24.1 uV	-1 uV	-3 uV	0.9 uV	.3
NRS *pain*	Primary-control	6.1	5.3	0.8	0.5	1.1	**<.0001**
	Secondary-control	5.9	5.3	0.6	0.3	0.9	**.0002**
SKIN ANALYSIS – BLOOD PERFUSION							
Basal flow	Primary-control	84.4 AU	40.4 AU	44 AU	39.2 AU	48.8 AU	**<.0001**
SKIN ANALYSIS – ERYTHEMA							
Haemoglobin average level	Primary-control	0.9 AU	0.8 AU	0.1 AU	0.1 AU	0.1 AU	**<.0001**
*Skin colour CIELab a**	Primary-control	11.3 AU	10.9 AU	0.37 AU	-0.1 AU	0.8 AU	.1

(Table continues on next page)

(Continuation Table 2)

Endpoint	Contrast	1st LSM[1]	2nd LSM[1]	ED	95% CI Lower	95% CI Upper	p-value
ELECTRICAL STAIR (SINGLE STIMULUS)							
	PDT: time						.89
	PDT: Period 2-Period 1	6.7 mA	6.1 mA	10.1%	-17.4%	46.6%	.47
	PTT: time						.49
	PTT: Period 2-Period 1	16.2 mA	16.7 mA	-2.7%	-15.3%	11.7%	.66
ELECTRICAL BURST (REPEATED STIMULUS)							
	PDT: time						.76
	PDT: Period 2-Period 1	1.5 mA	1.6 mA	-5.9%	-39%	45.4%	.76
	PTT: time						.4
	PTT: Period 2-Period 1	7.3 mA	7.8 mA	-6.4%	-21.6%	11.8%	.42
PRESSURE							
	PDT: time						.74
	PDT: Period 2-Period 1	15.3 kPa	15.4 kPa	-0.8%	-13.8%	14.2%	.91
	PTT: time						.11
	PTT: Period 2-Period 1	40.5 kPa	36.5 kPa	10.9%	-8.4%	34.2%	.25
COLD PRESSOR							
	PDT: time						.63
	PDT: Period 2-Period 1	5.7 s	5.2 s	9%	-22.5%	53.3%	.61
	PTT: time						.13
	PTT: Period 2-Period 1	23.2 s	18.8 s	23%	-0.3%	51.8%	.05
CPM							
	PDT: time						.66
	PDT: Period 2-Period 1	0.8 mA	0.2 mA	0.6 mA	-1.4 mA	2.6 mA	.52
	PTT: time						.33
	PTT: Period 2-Period 1	0.4 mA	0.8 mA	-0.4 mA	-2.6 mA	1.8 mA	.7

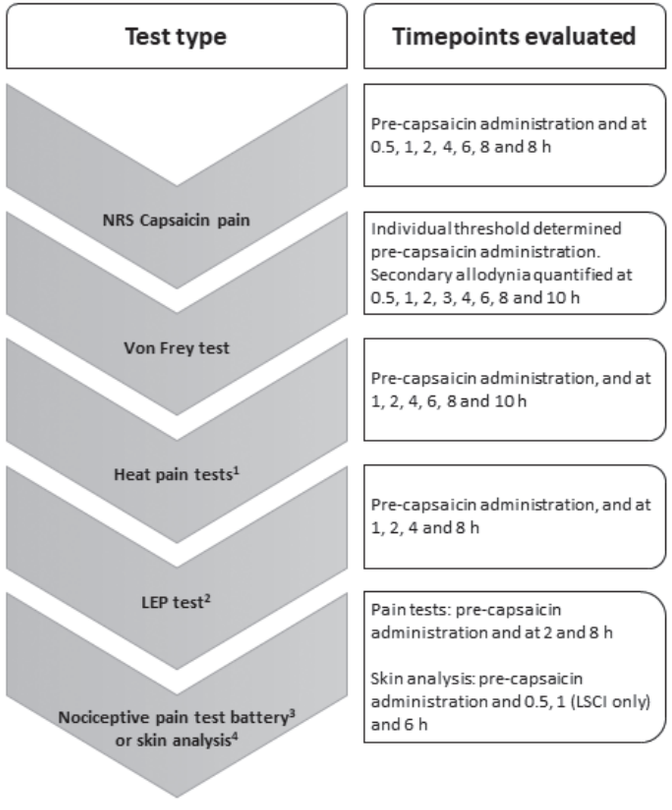
Selection of test results. 1: 'first' and 'second' LSM refers to the LSM of the first/second mentioned condition in the contrast (e.g., first LSM of primary – control refers to the LSM of the primary area). Positive ED values favor the first mentioned in the contrast (e.g. primary area, in the primary area – control area contrast) and vice versa. 'primary' is the area of skin treated with capsaicin, 'secondary' the area surrounding the treated (i.e., primary) area (both on the dominant arm); 'control' is the area on untreated skin (on the non-dominant arm). The contrast 'time' describes whether LSMs for specified test differed significantly over-time. 95% CI: 95% confidence Interval, CPM: conditioned pain modulation, ED: Estimate Of Difference, LSM: least square means, mm^2: square millimeters, MPQ: short-form McGill pain questionnaire, N2: maximum negative peak between 50 and 200 msec; P2: maximum positive peak between 150 and 500 msec; uV: microvolt. PDT/PTT: pain detection/tolerance threshold.

Table 3 Comparison of nociceptive test results between two CHDR studies using a capsaicin model.

Time (h) or contrast	1% capsaicin ethanolic solution (i.e., current) study N=10		Capsaicin cream formulation study [16] N=18	
	PDT (95% CI)	PTT (95% CI)	PDT	PTT
PRESSURE PAIN (kPa)				
1	-	-	11.4	41.8
2	-	-	11.0	42.3
3	15.17 (13.4 – 17.1)	37.6 (34 – 41.5)	12.2	44.5
4	-	-	12.6	42.4
6	-	-	11.5	43
9	15.5 (13.7 – 17.5)	39.3 (35.6 – 43.5)	-	-
10	-	-	12.6	41.4
COLD PRESSOR PAIN (S)				
1	-	-	4.0	23.0
2	-	-	3.9	23.5
3	5.6 (3.8 – 8.3)	22 (18.7 – 25.8)	3.7	22.9
4	-	-	3.8	22.2
6	-	-	3.1	23.7
9	5.2 (3.5 – 7.7)	19.9 (16.9 – 23.4)	-	-
10	-	-	3.4	21.9
ELECTRICAL STAIR PAIN (SINGLE STIMULUS, mA)				
1	-	-	6	22.3
2	-	-	5.1	21.8
3	6.4 (5.3 – 7.7)	16.3 (14.9 – 18.7)	6.5	21.7
4	-	-	6.3	21.8
6	-	-	5.6	22.2
9	6.3 (5.2 – 7.7)	16.7 (14.9 – 18.2)	-	-
10	-	-	5.3	22

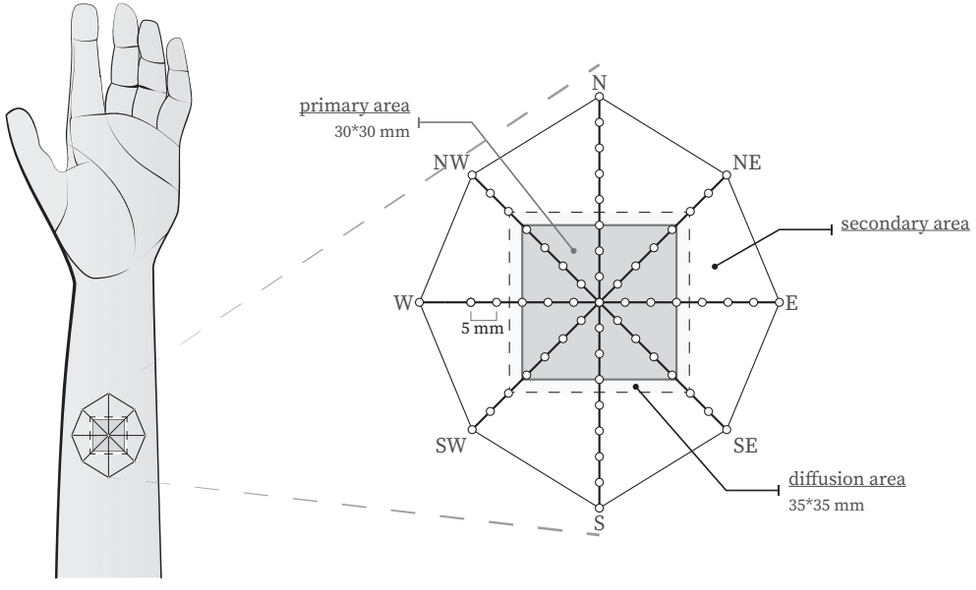
Comparison of PDT and PTTs per time point between current study that evaluated the 1% capsaicin ethanolic formulation, and the previous study that evaluated the 1% capsaicin cream formulation (Siebenga et al., 2020). [16] Data are presented as LSM's, including 95% CI's between parentheses for the current study. Statistical analysis of potential period effects (i.e., difference between LSM's in period 2 versus period 1) are included for the current study as well, presented in . For the previous study), data of the period in which subjects received placebo are included (reference [16]). '-': pain test not performed at respective timepoint for that study, 95% CI: 95% Confidence Interval, h: hour, kPa: kilopascal, LSM: least square means, mA: milliamperes, n : number of subjects, NA: not applicable, PDT/PTT: pain detection/tolerance threshold, s: seconds.

Figure 1 Order of assessments. Refer to the methods section for details of test procedures. Timepoints are protocol time (in hours) post-capsaicin administration, unless stated otherwise. 1: First heat pain test on capsaicin-treated skin followed by heat pain test on untreated skin. 2: First on treated area (i.e., primary area on dominant arm), followed by the untreated area (non-dominant arm) and the secondary area (i.e., surrounding primary area on dominant arm). 3: order: pressure pain test, electrical burst pain test, electrical stair pain test, cold pressor pain test, electrical stair pain test (repeat for evaluation of CPM). 4: First on capsaicin-treated area, then on control area (contralateral area on non-dominant arm). At coinciding timepoints first LSCI followed by MSCI.



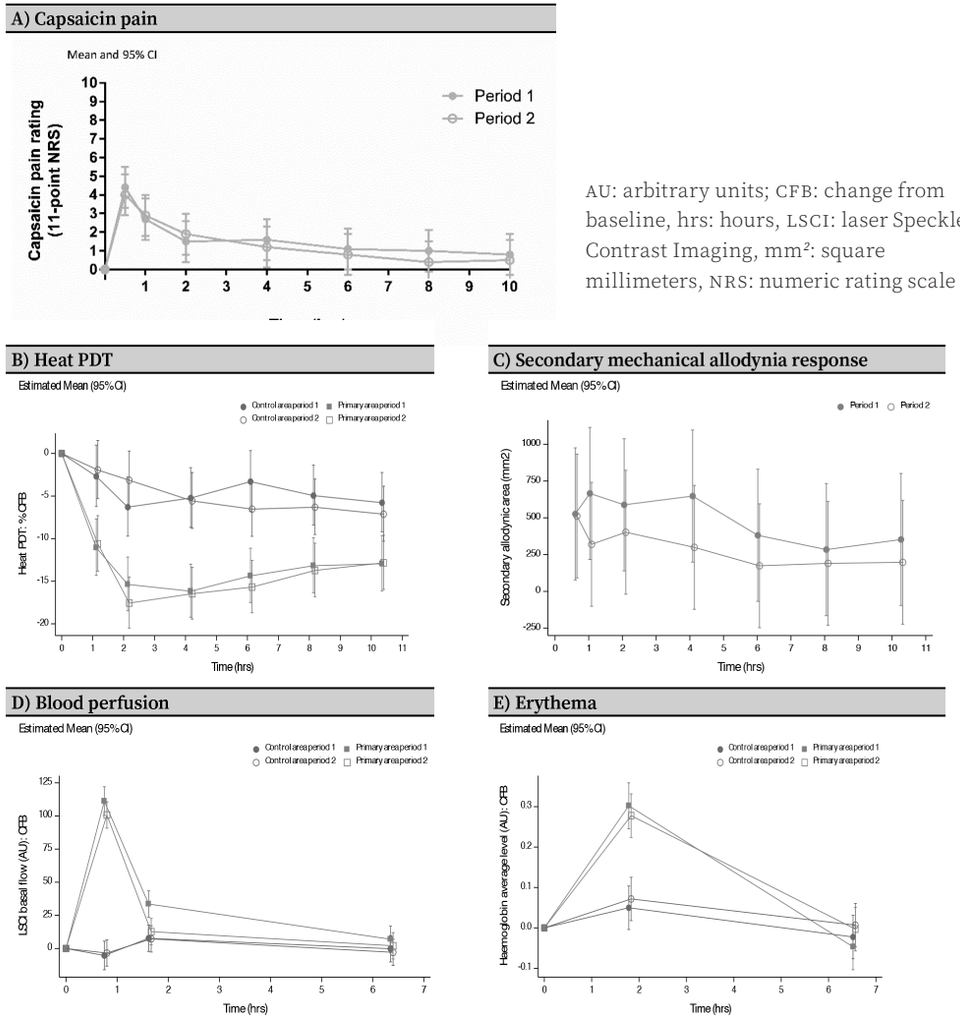
CPM = Conditioned Pain Modulation, h: hour, LSCI: Laser Speckle Contrast Imaging, MSCI: Multispectral Imaging, NRS: 11-point numeric rating scale.

Figure 2 Illustration of secondary mechanical allodynia assessment. Eight spokes divided a circle equally were drawn on the volar forearm to quantify the area of secondary allodynia. Assessment started on the north spoke at the most outer point from the center of the primary area (#1) and moved to the middle with steps of 5 mm. Once sensation changed from nearly painful to painful, that point was determined to be the border of the allodynic area. This assessment was repeated for all spokes in clockwise fashion. The allodynic area was quantified in mm2 using individual values for each spoke. A 5 mm border zone for each spoke surrounding the primary area (i.e. the 3×3cm capsaicin application area) was employed to minimize the risk of reporting false positive effects.



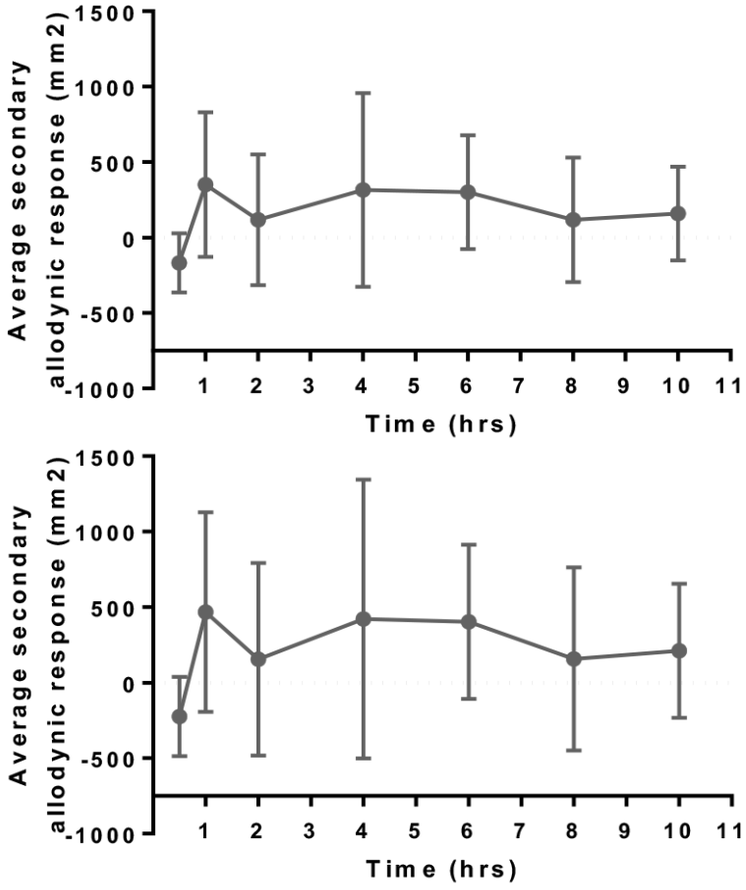
N: North, NE: Northeast, E: East, SE: Southeast, S: South, SW: Southwest, W: West, NW: Northwest, mm: millimeters. *The authors wish to thank Folkert van Meurs for illustrating this Figure.*

Figure 3 Overview of change from baseline time profiles. Effect-time profiles over the day for A) Subjective capsaicin pain rating using NRS, B) heat PDTs, C) secondary mechanical allodynia response, D) blood perfusion (i.e., basal blood flow), E) erythema (i.e., average haemoglobin level). Data are presented as estimated means with 95% confidence intervals. 'primary area' describe effects on the capsaicin treated area (i.e., on the 3×3cm area on the dominant arm), 'control area' describe the effect on untreated skin (i.e., on the contralateral area of where capsaicin was applied, on the non-dominant arm) 'period 1/2' describe the study period in which the observations were made.



AU: arbitrary units; CFB: change from baseline, hrs: hours, LSCI: laser Speckle Contrast Imaging, mm²: square millimeters, NRS: numeric rating scale

Figure 4 Intra-subject (within-subject) variability of the secondary allodynic response per measured time point. Data represented as mean difference in response between Period 1 – (minus) Period 2, with 95% confidence intervals. A) response for all subjects included in analysis (n=8); B) response for subjects defined as responder (n=6). Positive values indicate response was greater in Period 1; negative values indicate the response was greater in Period 2.



hrs: hours, mm²: square millimeters