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Sensory defunctionalization induced by 8% topical capsaicin treatment in a model of ultraviolet-B-induced cutaneous hyperalgesia.

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Abstract

Subpopulations of primary nociceptors (C and Aδ-fibers), express the TRPV1-receptor for heat and capsaicin. During cutaneous inflammation, these afferents may become sensitized, leading to primary hyperalgesia. It is known that $TRPV1^+$-nociceptors are involved in heat hyperalgesia, however their involvement in mechanical hyperalgesia is unclear. This study explored the contribution of capsaicin-sensitive nociceptors in the development of mechanical and heat hyperalgesia in humans following ultraviolet-B (UVB) irradiation.

Skin areas in 18 healthy volunteers were randomized to treatment with 8% capsaicin/vehicle patches for 24 hours. After patches removal, one capsaicin treated area and one vehicle area were irradiated with 2xMED (minimal erythema dose) of UVB. 1, 3 and 7 days post-UVB exposure, tests were performed to evaluate the development of UVB-induced cutaneous hyperalgesia: thermal detection and pain thresholds, pain sensitivity to supra-threshold heat stimuli, mechanical pain threshold and sensitivity, touch pleasantness, trans-epidermal water loss (TEWL), inflammatory response, pigmentation and micro-vascular reactivity. Capsaicin pretreatment, in the UVB-irradiated area (Capsaicin+UVB area), increased heat pain thresholds ($P<0.05$), and decreased supra-threshold heat pain sensitivity ($P <0.05$) 1, 3 and 7 days post-UVB irradiation, while mechanical hyperalgesia resulted unchanged ($P>0.2$). No effects of capsaicin were reported on touch pleasantness ($P=1$), TEWL ($P=0.31$), inflammatory response and pigmentation ($P>0.3$) or micro-vascular reactivity ($P>0.8$) in response to the UVB-irradiation. 8% capsaicin-ablation predominantly defunctionalizes $TRPV1^+$-expressing cutaneous nociceptors responsible for heat pain transduction, suggesting that sensitization of these fibers is required for development of heat hyperalgesia following cutaneous UVB-induced inflammation but they are likely only partially necessary for the establishment of robust primary mechanical hyperalgesia.

1. Introduction

Several subpopulations of primary afferents (C and Aδ-fibers) express the transient receptor potential (TRP-) channel V1 (Tominaga et al. 1998; Usoskin et al. 2015; Li et al. 2016; Hockley et al. 2018) which is involved in the transduction of heat nociception, chemo-nociception and pruriception (Anand and Bley 2011; Ross 2011).

Under perturbed circumstances, e.g. during skin inflammation or in neuropathic conditions, cutaneous primary nociceptors may become sensitised and cause primary hyperalgesia (Ali et al. 1996; Meyer 2006). Cutaneous inflammation, for instance following a sunburn, is often associated with both pronounced primary mechanical and heat hyperalgesia (Gustorff et al. 2004a, b; Sycha et al. 2005). Notably, mechanical nociception rely predominantly on populations of peripheral C- and Aδ-nociceptors which do not express TRPV1 receptor (Treede et al. 1998; Slugg et al. 2000; Magerl et al. 2001). These include MRGPRD$^+$ non-peptidergic C-fibers and type-I Aδ-nociceptors (Treede et al. 1998; Ringkamp et al. 2001; Zylka et al. 2005; Usoskin et al. 2015). The heat hyperalgesia seems to depend on sensitisation of TRPV1$^+$ nociceptors, however the potential contribution of TRPV1$^+$ fibers to development of primary mechanical hyperalgesia in humans is unclear and was the primary focus of the present study. While the majority of nociceptors are undoubtedly polymodal to some extent, rodent data suggest separate pathways for mechanical as well as heat nociception in inflammation-induced stages of hyperalgesia (Cavanaugh et al. 2009; Ross 2011; Zhang et al. 2013). In fact, mechanical and heat hyperalgesia associated with inflammation are in mice been selectively suppressed by ablation of MRGPRD$^+$/TRPV1$^-$ or TRPV1$^+$ fibers respectively, indicating that the neuronal substrates for the evoked heat and mechanical hyperalgesia may be distinct (Cavanaugh et al. 2009; Ross 2011; Zhang et al. 2013). The existence of distinct nociceptive fibers conveying heat and mechanical stimuli has been also demonstrate by a study conducted by Wang et al, showing that neurons expressing the mechanosensitive PIEZO1 channel are distinct from the population of neurons expressing the TRPV1 receptor (Wang et al. 2019). So far the existence of these two distinct neuronal populations has not been studied in details in humans.

It is well known that prolonged topical administration of high-concentration (>1%) capsaicin causes a temporary, profound epidermal denervation and sensory defunctionalisation in humans (Magerl et al. 2001; Henrich et al. 2015; Landmann et al. 2016; Andersen et al. 2017b). Hitherto, topical 8% capsaicin administration has shown to be a therapeutic option in the treatment of neuropathic pain patients (Backonja et al. 2008; Mou et al. 2013; Maihöfner and Heskamp 2014; Üçeyler and Sommer 2014; Landmann et al. 2016). The psychophysical deficiencies induced by a 24h 8% capsaicin–ablation in humans correspond strongly with a predominant effect on TRPV1$^+$–cutaneous fibers and are supported by rodent data (Cavanaugh et al. 2009; Zhang et al. 2013).

Topical administration of capsaicin induces reversible dermo-epidermal denervation and sensory loss (Nolano et al. 1999b; Dworkin et al. 2007; Gooding et al. 2010), by a 80% decrease in epidermal nerve fibre density 1

week after 1 h application of 8% capsaicin (Kennedy et al. 2010; Mainka et al. 2016), as also confirmed by studies on skin biopsies conducted after application of high-concentration capsaicin, or prolonged treatment (Nolano et al. 1999a; Ragé et al. 2010; van Neerven and Mouraux 2020)

In a recent human study, application of an 8% capsaicin patch for 1 h gave rise to a modest desensitization to warmth and heat stimuli that lasts for a few days (Lo Vecchio et al. 2018), while a 1 hour application only evokes prolonged sensory desensitization on the warmth threshold in neuropathic pain patients (Landmann et al. 2016; Mainka et al. 2016; Lo Vecchio et al. 2018). Prolonged (24 h) topical application of 8% capsaicin in healthy subjects, however, induces robust epidermal denervation and defunctionalization (Magerl et al. 2001; Henrich et al. 2015; Andersen et al. 2017b) with changes in warmth detection and heat pain threholds, histamine-induced itch and flare (Lo Vecchio et al. 2018).

This study aimed to investigate the effect of prolonged (24 h) capsaicin application on development of heat and mechanical hyperalgesia following a controlled UVB induced cutaneous inflammatory perturbation. The effect of capsaicin on UVB-induced erythema and pigmentation, as well as the changes in skin integrity were investigated.

It was hypothesised that the UVB-induced inflammatory provoked heat hyperalgesia would be inhibited in capsaicin-ablated skin, while development of mechanical hyperalgesia would only be minimally impacted.

2. Methods

2.1 Subjects and study design

The development of unspecific UVB-cutaneous inflammation and consequent neurogenic inflammation in a 8% capsaicin pre-treated area were explored in 18 healthy subjects (5 F, 13M, 24.5 ± 1 years). Exclusion criteria included pregnancy, lactation, previous or current musculoskeletal, neurologic or dermatologic diseases, tattoos in the forearm areas, as well as ongoing pain or medication intake. All subjects signed an informed consent and the regional Ethics Committee approved the protocol (N-20180034) in accordance to the Helsinki Declaration. The middle forearms of each subject were divided into 2 squared areas (4x4 cm) located 4 cm apart. Two of them were pre-treated with 8% capsaicin patches for 24 h, while the other two areas were treated with vehicle patches (Fig 1). Twenty-four hours after patches removal, one of the capsaicin treated areas as well as one of the vehicle areas were irradiated with 2XMED (minimal erythema dose) of UVB. At day 1, 3 and 7-post UVB exposure, quantitative sensory tests (QSTs) were conducted to assess the development of UVB induced cutaneous inflammation in the 8% capsaicin treated area compared to vehicle patch treatment (Fig 1). The study was divided in 6 sessions, over a period of 10 days. In session 1, two capsaicin patches and two vehicle patches were applied on the forearms for 24 h. In session 2, (24 h after the 1st session) the four patches were removed simultaneously, while in session 3 one of the capsaicin treated areas as well as one of the vehicle treated areas were irradiated with 2xMED. In session 4 (1 day after UVB irradiation) the first battery of sensory and vasomotor tests was conducted. These tests were repeated on day 3 after irradiation

(session 5) as well as on day 7 after irradiation (session 6). The QST tests, have been adapted from a standardized QST protocol (Rolke et al. 2006a, b), and were performed in each skin area following the UVB irradiation (Fig 1).

2.2 UVB irradiation

Approximately one week before the experiment, the minimal erythema dose (MED) for the UVB irradiation was determined for each participant using a calibrated UVB source (wavelength 290-320 nm; Saalmann Multitester, SBC-LT 400 Herford, Germany). The MED is defined as the minimum amount of UVB energy (J/cm^2) producing a clearly visible erythema in the skin 24 hours after UVB exposure. For MED determination, at the anterior surface of the right thigh of each participant, five circular skin spots (Ø = 2 cm) were irradiated with a series of increasing UVB doses ranging from 40 to130 mJ/cm^2. Following MED determination, two skin areas were irradiated by 2×MED. Irradiations of proximal/distal areas in the forearms were performed in a balanced, randomized manner. QSTs were conducted at day 1, 3 and 7 post-irradiation.

2.3 Transdermal capsaicin

Two squared areas (4x4 cm) on the forearms of each volunteer were treated with two capsaicin patches (8% transdermal patch, 'Qutenza', Astellas) or two vehicle patches (Demo patch, Astellas, containing the same formulation of the active patches but without the capsaicin component) for 24h. All patches were applied in compliance with the manufacturer's directions with the experimenter using nitrile gloves, and the application was randomized so that the two capsaicin patches were always placed in different forearms. The patches were covered with opaque medical tape in order to blind the subject to the active treatment (capsaicin vs vehicle) and left in place for 24 h after which they were removed. After patch application, the participants were provided with a template to report the capsaicin-evoked pain for the first 6 h following application on a numerical rating scale (NRS_{0-10}) from 0 to 10, with 0 indicating "no pain" and 10 indicating "worst pain imaginable". The participants were also instructed to report a retrospective score of mean and peak pain experienced during the 24h exposure. Both scores were reported for left and right arm.

The same concentration and duration of capsaicin administration applied in this study have previously been used in other studies (Henrich et al. 2015; Andersen et al. 2017a; Lo Vecchio et al. 2018).

2.4 Thermal Assessments

2.4.1 Warmth detection thresholds and heat pain thresholds

Warmth detection thresholds (WDT) were measured using a Pathway thermal sensory testing device (Medoc Ltd, Israel). A thermal stimulator probe of 3x3 cm was placed on the treated/control areas and increasing ramp stimuli of 1°C/s were delivered from a starting temperature of 32°C. The participant was instructed to press a button as soon as warmth or an increase in temperature was felt. Each assessment was repeated three times.

Heat pain thresholds (HPT) were measured with the same device used for the warmth detection thresholds assessment. The participant was instructed to press a button as soon as a painful sensation was felt. Each assessment was repeated three times.

2.4.2 Pain sensitivity to supra-threshold heat stimuli

Supra-threshold heat sensitivity (STHS) was measured using the same device described above. The participants were instructed to rate the average pain to two suprathreshold heat pain stimuli (starting and ending at 32°C with an increase and decrease of 5°C/s and 1 s plateau at 46°C).

2.5 Mechanical Assessments

2.5.1 Mechanical pain thresholds (MPT) and pain sensitivity (MPS)

Mechanical pain thresholds (MPT) were assessed using a set of seven pinprick stimulators (MRC Systems GmbH, Germany) each exerting different forces ranging from 8 to 512mN (Log2 scale). Starting with the lightest, each pin was applied at a rate of 2 s on and 2 s off and in ascending order until the subject reported a perception of sharpness or pain. The stimuli were applied in an area of 1 cm around the center of the 4x4 cm area, and the location was changed after each stimulation. In cases where the pain threshold was not reached with any of the seven stimulators, the value 1024 mN was recorded (this value correspond to the pin immediately above 512mN). The final threshold was calculated as the geometric mean of five consecutive series of ascending/descending stimuli.

Mechanical pain sensitivity (MPS) was assessed using the same set of pinprick stimulators. In this case the 1024mN was not used for the MPS evaluation. Starting with the lightest, each pin was applied in an ascending order on each area and the subject was asked to rate the pain using the same $NRS_{0\text{-}10}$ scale described for capsaicin-evoked pain (section 2.3). This process was repeated twice. The final MPS was calculated as the mean of the ratings of 2 consecutive series of the 7 pinprick stimulations.

2.5.2 Touch Pleasantness (TP)

Pleasant touch sensations were assessed using a standardized sensory brush (SENSELab Brush-05, Somedic AB, Hörby, Sweden) exerting a force of 200 to 400 mN. The brush stimuli consisted of three 2 cm in length strokes with an interval of 10 sec over the treated/control areas. The strokes were applied perpendicularly to the skin. After applying the strokes, the subjects were asked to report the sensation induced by the brush on a NRS scale labeled “very unpleasant” and “very pleasant” at the extremity and “neutral” at the center. The subjects were asked to keep their eyes closed during the procedure.

2.6 Trans-epidermal Water Loss (TEWL)

TEWL is a non-invasive and pain free measuring technique used to investigate changes in the skin barrier integrity in vivo. Before the measurement, all subjects were seated in a temperature-humidity controlled room for 10 to 15 minutes. During the measurement, a 2x2 cm hollow probe, with two small humidity gauges and two thermometers (DermaLab, Cortex Technology, Denmark), was placed gently on the skin for 10-25 seconds. These collectively establish an exact humidity gradient in the 2 cm vicinity of the skin, representative for the point-loss of water through the epidermis. TEWL is measured in g of water/m^2/hour and is defined as the evaporation rate of water from the epidermal layer of the skin into the surrounding atmosphere via diffusion and evaporative processes (Thiele et al. 2003). The procedure is entirely non-invasive and not associated with any pain or discomfort.

2.7 Neurogenic inflammatory response and pigmentation

The development of erythema (redness) and skin pigmentation following the UVB irradiation were measured with a spectrometer designed for cutaneous use (ColorMeter, DSM II; Cortex Technology, Hadsund, Denmark). The spectrometer provides information of erythema and pigmentation based on the skin's light absorption for wavelengths corresponding to haemoglobin (erythema) and melanin (pigmentation). Erythema and pigmentation data were recorded at day 1, 3 and 7 after UVB irradiation and are expressed as Arbitrary Unit (A.U.).

2.8 Micro-vascular reactivity

The UVB-evoked cutaneous inflammatory reaction, (quantified by superficial blood perfusion), was measured by Full-Field Laser Perfusion Imaging (FLPI2, Moor Instruments, Axminster, Devon, UK). The device was placed 25 cm above the skin surface with an exposure time of 8.3 milliseconds and 160 units of gain. Images were obtained at day 1, 3 and 7-post UVB exposure for both forearms and the MoorFLPI2 Review V5.0 proprietary software was used for the analysis. The FLPI data were analysed using a region of interest (ROI) approach with ROIs equivalent to the predefined irradiated area. The mean and peak perfusion values from each ROI were extracted.

2.9 Statistical analysis

Data handling and calculation of mean and SEM were conducted in Excel. Statistical analyses were performed using SPSS (v25, IBM Corporation, NY, USA), while the graph plotting was realized using GraphPad Prism 8.1.2 (GraphPad Software Inc., CA, USA). For each parameter, the effect size has been reported as Cohen's d value for a within-subject design. Data from all assessments were normality tested using visual inspection and if unclear, the Shapiro-Wilks test was used. Main analyses were repeated measure analyses of variance (RM-ANOVAs) followed by Sidak post hoc tests. *P*-values ≤ 0.05 were considered statistically significant. RM-

ANOVAs were constructed with the factors *treatment* (placebo, capsaicin, UVB, and capsaicin+UVB) and *time* (day 1, 3, and 7). Data for the QSTs are also presented as Δ-values from the capsaicin, capsaicin+UVB and UVB conditions to their respective vehicle condition (demo patch) to provide a clearer overview of the results. For the analysis of the 6 hours pain rating after patch application RM-ANOVA was constructed with the factors *side* (left and right) and *time* (hour 1, 2, 3, 4, 5, and 6)

3. Results

Twenty subjects were recruited for the study, but only 18 were included, since one participant was excluded due to HPT values below the normative data (Yarnitsky et al. 1995), (mean HPT in the control area: 34.6 °C) and one subject chose to remove the patch prematurely, since this option had been clearly highlighted (should the capsaicin-evoked pain become unbearable) when the patches were applied, and was therefore excluded. The 18 remaining participants completed the procedures without safety issues during or after the study and well tolerated the prolonged administration of 8% capsaicin well.

The subjects experienced mild-to-moderate pain during the 6 h of capsaicin patch application with an average peak pain intensity occurring after 3 h of 5 ± 0.4 ($NRS_{0–10}$) for both left and right forearm. No difference was reported between left and right forearm for any of the time points ($F_{5,85}$= 0.37, Fig 2A).

Using the same scale, the averaged pain intensity for the 24 h administration rated retrospectively after patch removal was 4 ± 0.3 for the both forearms; while the peak pain was 7 ± 0.5 and 6 ± 0.5 for the left and right forearm respectively (Fig 2B).

3.1 Thermal Assessments

For WDT a treatment × time interaction ($F_{3.7,62.3}$=8.2, P<0.001) was present, with post-hoc testing showing that the UVB irradiation alone does not induce any decrease in WDT compared to vehicle (P>0.6) at any time point. The overall mean in the vehicle condition across the three time points was 35.8 ± 0.4.

Both capsaicin pre-treated areas (capsaicin and cap+UVB areas), showed increased WDT compared to both vehicle (effect size day 1, capsaicin: d=2.2; cap+UVB: d=1.6) and UVB (day1, capsaicin: d=2.1; cap+UVB: d=1.6) areas (P<0.001, Fig 3A, Fig 4A) at any time point and no difference between these 2 conditions (capsaicin and cap+UVB areas), was reported at any time points (P>0.1). At day 1 the WDT was 35.4 ± 0.3 °C in the vehicle area versus 35.5 ± 0.3 °C in the UVB area, 41.9 ± 0.7 °C in the capsaicin area and 40.3 ± 0.8 °C in the cap+UVB area.

For HPT a treatment × time interaction ($F_{6,102}$=25.3, P<0.001) was present, with post-hoc testing showing that the UVB irradiation alone induced a statistically significant decrease in the HPT (P<0.05, day1, d=1.6) compared to vehicle, from 42 ± 0.5 °C at day 1 in the vehicle area to 39 ± 0.3 °C in the UVB area. In this area, the HPT was normalized after 7 days (P=0.79, Fig 3B, Fig 4B).

Capsaicin alone induced an increase in HPT compare to vehicle (P<0.001, day1, d=2.3) at any time point.

In the capsaicin+UVB area, the capsaicin induces an increase in HPT compare to vehicle (P<0.05, day1, d=1.6) at any time point, indicating that capsaicin pre-treatment seems to prevent the thermal hyperalgesia induced by UVB treatment alone, as confirmed by a statistical difference between the two UVB treated areas (UVB and cap+UVB areas, P=0.01, day1, d=3.4). At day 1 the capsaicin effect was higher compared to the cap+UVB (P<0.05, d=0.9), indicating that in the cap+UVB area, capsaicin induces a level of thermal hypoalgesia almost equal to the one induced by capsaicin pre-treatment alone. At day 1 the HPT was 48.3 ± 0.4 °C in the capsaicin area and 46.5 ± 0.6 °C in the cap+UVB area.

For the STHS a treatment × time interaction ($F_{6,102}$=13,7, P<0.001) was present, with post-hoc testing showing that the UVB irradiation alone induced a statistically significant increase compared to vehicle (P<0.01, day 1, d= 1.8; Fig 3C, Fig 4C) from 4.2 ± 0.5 at day 1 in the vehicle area to 6.3 ± 0.5 in the UVB area. In this area, the STHS was normalized after 7 days (P=0.93, Fig 3C, Fig 4C).

In the capsaicin area, capsaicin induced a statistically significant decreased STHS compare to vehicle (P<0.05, day 1, d=1.6; Fig 3C, Fig 4C) at any time point.

In the capsaicin+UVB area, the capsaicin induces a decrease in STHS compare to vehicle (P<0.01, day 1, d=1.3) at any time point, confirming that capsaicin pre-treatment seems to prevent the thermal hyperalgesia induced by UVB treatment alone as confirmed by a statistical difference between the two UVB treated areas (UVB and cap+UVB areas, P<0.05, day1, d=2.5). At day 1 the STHS was 4.2 ± 0.5 in the vehicle area versus 1 ± 0.4 in the capsaicin area and 1.7 ± 0.6 in the cap+UVB area. No difference was reported between the two capsaicin pre-treated areas at any time point (P>0.5).

Hence, capsaicin pre-treatment seems to inhibit the development of thermal hyperalgesia induced by UVB treatment alone.

3.2 Mechanical assessments

For the MPT a treatment × time interaction ($F_{2.9,49.3}$=13, P<0.001, Fig 3D, Fig 4D) was present, with post-hoc testing showing that the UVB irradiation alone induced a significant decrease in MPT compared to vehicle (P<0.001, day 1, d=0.8; Fig 3D, Fig 4D), from 284.9 ± 69.9 mN at day 1 in the vehicle area to 78.5 ± 20.2 mN in the UVB area, that was normalized after 7 days (P=0.97).

In the capsaicin area, capsaicin tended to induce a slight, but not significant, increase in MPT compared to vehicle (P>0.3) at any time point. At day 1 the MPT in the capsaicin area was 414 ± 88.8 mN.

In the capsaicin+UVB area, once the area has been pre-treated with capsaicin, the UVB did not induce any changes in MPT compared to vehicle at any time point (P>0.22, at day 1 the MPT in the capsaicin+UVB area was 206.4 ± 50 mN), indicating that capsaicin pre-treatment seems to partially prevent the mechanical hyperalgesia induced by UVB treatment alone as confirmed by a statistical difference between the two UVB treated areas (UVB and cap+UVB areas, P<0.01, day 1, d=0.8) that normalize after 7 days (P=0.7). Moreover,

also between the two capsaicin pre-treated areas (cap and cap+UVB areas) there was a difference in MPT ($P<0.05$, day 1, d=1) that was normalized after 7 days (P=0.43).

For the MPS a treatment × time interaction ($F_{3.8,65}=7.3$, $P<0.001$, Fig 3E, Fig 4E) was present, with post-hoc testing showing that the UVB irradiation alone induced significantly increased MPS compared to vehicle ($P<0.01$, day 1, d=1.1; Fig 3E, Fig 4E), from 0.6 ± 0.1 at day 1 in the vehicle area to 1.2 ± 0.2 in the UVB area, that was normalized after 7 days P=0.12.

In the capsaicin area no changes in MPS compared to vehicle ($P>0.2$) was found at any time point. The overall mean in the vehicle condition across the three time points was 0.42 ± 0.1.

In the capsaicin+UVB area, the UVB does not change the MPS compared to vehicle ($P>0.5$) at any time point, indicating that capsaicin pre-treatment seems to inhibit the increase in mechanical sensitivity induced by UVB treatment alone as confirmed by a statistical difference between the two UVB treated areas (UVB and cap+UVB areas, day 1, d=1, $P<0.01$) that normalize after 7 days (P=1). Moreover, also between the two capsaicin pre-treated areas (cap and cap+UVB areas) there was a difference in MPS ($P<0.05$, d=0.8) that was normalized after 3 days (P=1).

The analysis of the TP does not show any effect of time or treatment and the treatment × time interaction was also not significant (P=0.1, Fig 3F, Fig 4F), indicating that neither capsaicin nor UVB have any effect on touch perception. The overall mean in the vehicle condition across the three time points was 64 ± 2.7.

Hence, capsaicin pre-treatment only partially prevented the mechanical hyperalgesia induced by UVB treatment alone.

3.3 Erythema and Pigmentation

For the erythema, the treatment × time interaction ($F_{6,102}=9.2$, $P<0.001$) was present, with post-hoc testing showing that capsaicin does not induce erythema compared to vehicle ($P>0.3$) at any time point. The overall mean in the vehicle condition across the three time points was 8.6 ± 0.5 AU.

In the two conditions cap+UVB and UVB, the UVB irradiation induced an intense erythema compared to vehicle ($P<0.001$, day 1, cap+UVB: d=4.6; UVB: d=4) at any time point. No differences between these two conditions were present at any time point ($P>0.9$). At day 1 the erythema was 8.7 ± 0.5 AU in the vehicle area versus 17.2 ± 0.6 AU in both UVB and cap+UVB area. In these two last conditions the erythema decreased over time ($P<0.05$, Fig 5A).

For the pigmentation, the treatment × time interaction ($F_{3.8,65.3}=24$, $P<0.001$) was present, with post-hoc testing showing that capsaicin does not have an effect on skin pigmentation compare to placebo ($P>0.9$) at any time point. The overall mean in the vehicle condition across the three time points was 31.4 ± 1 AU. In the two conditions, cap+UVB and UVB, the UVB irradiation induced an increased skin pigmentation compare to vehicle, starting 3 days after irradiation ($P<0.001$, day 3, cap+UVB: d=2.2; UVB: d=1.8; Fig 5B). No

differences between these two conditions were present at any time point (P>0.9). Hence, capsaicin pre-treatment did not have any effect on erythema and pigmentation induced by UVB irradiation.

3.4 Trans-epidermal Water Loss Assessments

The analysis of the TEWL does not show any effect of time or treatment and the treatment × time interaction was also not significant (P=0.31, Fig 5C), indicating that neither capsaicin nor UVB have any effect on the integrity of the skin barrier. The overall mean in the vehicle condition across the three time points was 9.8 ± 0.8 g m^{-2} h^{-1}. Hence, neither capsaicin pre-treatment nor the UVB-irradiation altered TEWL.

3.5 Micro-vascular reactivity

For both mean and peak superficial perfusion, a treatment × time interaction ($F_{2;33.4}$=54,5, P<0.001) was present, with post-hoc testing showing that capsaicin did not have any effect on the development of inflammatory flare compared to vehicle (P>0.8, Fig 5D, data of peak are not showed) at any time point. In the two conditions, cap+UVB and UVB, the UVB irradiation induced an intense micro-vascular response compared to vehicle (day1, mean: cap+UVB: d=2.2, UVB: d=2.1; peak: cap+UVB: d=2.3, UVB: d=2; P<0.05) at any time point. At day 1 the mean and peak perfusion were 119 ± 7 AU and 245 ± 14 AU respectively in the vehicle area versus 546 ± 49 AU and 1103 ± 101 AU respectively in the UVB area, and 556 ± 50 AU and 1124 ± 93 AU respectively in the cap+UVB area. No differences between these two conditions were present at any time point (P>0.7). In these two last conditions the inflammatory flare decreased over time (P<0.01, Fig 5D). Hence, capsaicin pretreatment had no impact on the inflammatory flare generated by the UVB-irradiation.

4. Discussion

This study showed that capsaicin pre-treatment decreased the thermal hyperalgesia induced by UVB evoked inflammation, whereas the mechanical pain sensitization was only partially affected.

4.1 UVB induced hyperalgesia

The UVB model has been extensively used in both animals (Bishop et al. 2007, 2010) and humans (Sycha et al. 2003, 2005; Gustorff et al. 2004a, b, 2013; Bishop et al. 2009; Lo Vecchio et al. 2018), and has been applied, in clinical context, for assessing the efficacy and mechanism of action of anti-inflammatory drugs and analgesics (Sycha et al. 2003; Gustorff et al. 2004b, a; Mørch et al. 2013). This model is characterized by increased mechanical and thermal pain sensitivity as well as increased response to suprathreshold stimuli which peak about 24 h post-irradiation (Hoffmann and Schmelz 1999; Benrath et al. 2001; Gustorff et al. 2004b; Harrison et al. 2004; Bishop et al. 2009). In line with these findings, in the present study, the peak of cutaneous hyperalgesia for the UVB model was found to occur 24-48 h after the irradiation.

4.2 Capsaicin-induced nociceptors defunctionalization

Upon application, topical capsaicin results in burning pain, erythema, hyperalgesia and allodynia (Malmberg et al. 2004), but after prolonged exposure to high concentration of capsaicin, the TRPV1 receptors enter in a refractory desensitization state (Burks et al. 1985; Lee et al. 1991; Comunanza et al. 2011; Fattori et al. 2016). Evidences suggested that this desensitization includes depletion of neuropeptides such substance P (Fattori et al. 2016) and a massive influx of Ca^{2+} leading to depolymerization of cytoskeletal components and direct inhibition of mitochondrial respiration, resulting in impaired local nociceptors function (defunctionalization) (Anand and Bley 2011; O'Neill et al. 2012; Andersen et al. 2017a).

4.3 Thermal sensitivity

In this study, the WDT, was profoundly desensitized by capsaicin. This finding is in line with earlier studies conducted using high-concentration capsaicin (Malmberg et al. 2004; Mainka et al. 2016; van Neerven and Mouraux 2020). It is known that the TRPV1 receptor responds to thermal stimuli ≥ 43 °C, which is above the normal perception temperature for warmth stimuli (Lumpkin and Caterina 2007; Lo Vecchio et al. 2018). However, another TRP family member, the TRPV3 receptor, can also be activated by heat and it is responsible for detecting warmth in the innocuous range, about 32-39°C (Xu et al. 2002; Cheng et al. 2007; Lumpkin and Caterina 2007; Lo Vecchio et al. 2018), explaining the observed results. Recent evidence suggests that the two receptors, TRPV1 and TRPV3, are co-expressed on warmth C-fibers and interact to form functional heteromeric channels (Smith et al. 2002; Xu et al. 2002; Cheng et al. 2007; Lumpkin and Caterina 2007; Lo Vecchio et al. 2018). Such heteromerization allows sensory neurons to express receptors with a range of responses wider than those expressed by the individual homomeric receptors (Caterina et al. 1999; Smith et al. 2002; Cheng et al. 2007). In this study we found that UVB alone does not have any effect on warm detection. Recent evidence shows that the TRPV3 receptor is not expressed solely in neurons but it is also expressed in non-neuronal cell types such as epidermal keratinocytes where it is implicated in the cutaneous inflammatory process, and apoptotic cell death (Szöllősi et al. 2018). In spite of this evidences, we found no effect on WDT of UVB alone, suggesting that the activation of TRPV3 receptor on keratinocyte following UVB irradiation is not sufficient to induce a decrease in WDT as showed by the present findings.

The UVB irradiation alone caused a decreased in HPT and an increased STHS up to 3 days after irradiation, confirming that UVB induces thermal hyperalgesia in the irradiated area (Hoffmann and Schmelz 1999; Bishop et al. 2009; Dawes et al. 2011; Weinkauf et al. 2013; O'neill et al. 2015). The release of inflammatory mediators, subsequent to UVB irradiation, can sensitize TRPV1channels, inducing an axonal hyper excitability correlated with heat hyperalgesia (Snyder 1975; Wilgus et al. 2002; Kienzler et al. 2005; Sycha et al. 2005; Weinkauf et al. 2013; O'neill et al. 2015). The key role of TRPV1 receptors in the development of thermal hyperalgesia is underlined by the consideration that the development of UVB induced heat hyperalgesia was

completely inhibited in capsaicin-ablated skin. In the cap+UVB area, capsaicin induced almost the same hypoalgesic effect induced by capsaicin alone, despite the fact that results on erythema indicated robust inflammation in both areas. The significant heat hypoalgesia showed by the increase in the HPT, and remarked by the decrease in STHS after capsaicin treatment, is mediated by the direct defunctionalization of TRPV1-expressing nerve fibres induced by the prolonged capsaicin treatment (Anand and Bley 2011), and the fiber populations affected are likely to be polymodal-C- and type-II Aδ-fibers (LaMotte and Campbell 1978; Treede et al. 1998; Dubin and Patapoutian 2010; Lo Vecchio et al. 2018). This is in line with the results published by Landmann et al, which reported thermal hypoesthesia at day 1 and 3 after 1 hour topical capsaicin treatment which normalized 1 week after treatment (Landmann et al. 2016). In the present study however, both WDT and HPT were still statistically increased after 1 week, and this dissimilarity may be ascribed to the different time application (24h vs 1h) of the 8% capsaicin patches.

4.4 Mechanical sensitivity

Touch sensitivity is mediated by Aβ- and C-tactile afferents which do not expresses the TRPV1 receptor on their surface (Olausson et al. 2010; Akiyama and Carstens 2013; Usoskin et al. 2015; Lo Vecchio et al. 2018). This explain the current results showing that touch sensitivity was unaffected by all provocation. The lack of effect of capsaicin on these outcomes is in line with other studies reporting the same conclusions (Kennedy et al. 2010; Mainka et al. 2016). Moreover, the Aβ-afferents responsible for tactile sensitivity largely terminate just below the dermo-epidermal junction and may only be reached by very low concentrations of capsaicin (Lo Vecchio et al. 2018). No tactile allodynia was induced by UVB irradiation. It is possible that the UVB dosage (2xMED) used in this study was too low for tactile allodynia to develop.

The UVB irradiation alone induced a statistically decreased of MPT accompanied by an increased mechanical pain sensitivity up to 3 days after irradiation, confirming that UVB induced inflammation leads to development of mechanical hyperalgesia in the irradiated area. These data are in line with other evidence showing decreased mechanical thresholds and increased sensitization of high-threshold mechanosensitive C-nociceptors within the area of UVB irradiation (Bishop et al. 2007; O'neill et al. 2015). It is well known that mechanical nociception rely predominantly on populations of polymodal C- and Aδ-nociceptors (Treede et al. 1998; Slugg et al. 2000; Magerl et al. 2001; Lo Vecchio et al. 2018). Interestingly, we found that UVB-induced mechanical hyperalgesia was partially inhibited in capsaicin-ablated skin, but the overall effect of capsaicin ablation on mechanical sensitization was only partial, indicating that mechanical hyperalgesia is only partially driven by $TRPV1^+$ fibers. This partial desensitization may suggest that only a subpopulation of the pinprick pain-conveying fibers is affected. A study published by Magerl et al., showed that injection of capsaicin induces secondary mechanical hyperalgesia to pinprick mediated by Aδ-nociceptors that are insensitive to capsaicin, and specifically two classes of A-fibres that together represent two-third of all A-fibres: the Type I A-fibre mechano-heat-sensitive nociceptors (Type I AMHs) and the high-threshold mechanoreceptors

(HTMs) (Magerl et al. 2001; Kennedy et al. 2010; van Neerven and Mouraux 2020). Moreover, some of the primary afferents innervating the skin express the Mas-related G-protein receptor D (MrgprD), which is mechano-receptive but is not co-expressed with the TRPV1 receptor (Zylka et al. 2005; Usoskin et al. 2015; Lo Vecchio et al. 2018). Accordingly, this MrgprD$^+$ cutaneous fiber may carry the residual pinprick pain sensitivity currently observed (Tiwari et al. 2016; Lo Vecchio et al. 2018). In a recent study, the role of β-alanine, a MrgprD$^+$agonist, as a potential model of non-histaminergic itch has been investigated (Christensen et al. 2019) and in further study this method could be used to validate the contribution of MrgprD$^+$ neurons on mechanical hyperalgesia.

4.5 Skin integrity, erythema, pigmentation and micro-vascular reactivity

Disruption of the skin barrier function has been objectively measured by quantifying the trans-epidermal water loss. TEWL is also known as "insensible water loss" as it is a mainly autonomic process and is used as an indicator of decreases in the functional integrity of the stratum corneum (Berardesca et al. 2013; Akdeniz et al. 2018), e.g., caused by pathological conditions such as skin inflammation or atopic dermatitis, by certain chemicals or physical insult (such as UVB-irradiation and other xenobiotics). These conditions will result in an increased trans-epidermal water loss (Thiele et al. 2003), that would indicate a loss of skin barrier function, accompanied by dryness of the skin and visible surface scales (Zhou et al. 2018). In particular, UV radiations exposure, induces the release of several effector molecules, including cytokines and prostaglandins, which initiate a non-neurogenic inflammatory response, accompanied by erythema and hyperplasia (Haratake et al. 1997). In the current study, we found that capsaicin did not have any effect on TEWL indicating that TRPV1$^+$ fibers are not involved in the disruption of the integrity of the skin barrier. Moreover, we found that also UVB irradiation, alone or in combination with capsaicin, did not show effect on the integrity of the stratum corneum. These results were somewhat unexpected, since has been demonstrated that UVB radiation is known to induce skin oxidative perturbations, as showed in other studies (Haratake et al. 1997; Meguro et al. 2000; Thiele et al. 2003; Jiang et al. 2006). In particular, Haratake et al., demonstrated in hairless mice that the extent of TEWL increase was UVB dose-dependent (Haratake et al. 1997), while Jiang et al, demonstrated that the TEWL increase started 3 days after irradiation, peaked 4 days post-irradiation, and start to decline at day 5 post-irradiation (Jiang et al. 2006). A possible explanation for these different results might be the diverse MEDs used in the different studies. Thiele, Haratake and Jiang, found increases TEWL with the use of 3×MED, 4.5×MED and 7.5 MED, respectively, while in our study we only used 2×MED (Haratake et al. 1997; Thiele et al. 2003; Jiang et al. 2006). Only Meguro et al, demonstrated an increase TEWL using 2×MED UVB in rats (Meguro et al. 2000). Based on all these results, it is possible to argue that mostly doses of 3×MED or above are required to induce measurable human skin barrier disruption.

The most obvious effects of UVB exposure are skin erythema (sunburn) and increased pigmentation (tanning) (Hönigsmann 2002). The erythema tends to fade over few days and it is followed by skin tanning (Hönigsmann

2002). As expected, 24 h after irradiation, UVB induced an intense erythema accompanied by an increased pigmentation which started three days after irradiation. Capsaicin had no effects on erythema nor pigmentation, suggesting that both the erythema and hyperpigmentation are mediated by non-neurogenic inflammatory responses induced by UVB irradiation (Yamaguchi et al. 2007), or at least without any involvement from $TRPV1^{+}$-fibers.

As expected, the UVB irradiation induced an intense hyperemia that decreased over time, in line with several studies reporting an increase in blood flow in the irradiated area (Benrath et al. 2001; Bishop et al. 2009; Lo Vecchio et al. 2014; Lo Vecchio 2015; Andersen et al. 2018). The non-neurogenic inflammatory response induced by UVB irradiation is caused by release of vasoactive inflammatory mediators that act directly on the vasculature (Angst et al. 2008; Lo Vecchio 2015).

In the present study, capsaicin pre-treatment did not have any effect on the development of neurogenic inflammation, confirming the non-neurogenic origin of the hyperemia induced by UVB irradiation. Moreover, recent studies have found an increased production of nitric oxide (NO) by cutaneous keratinocytes in response to UVB-irradiation which may be directly involved in vasodilatation and cutaneous response following irradiation (Clydesdale et al. 2001; Rhodes et al. 2001; Clough and Church 2002; Ayajiki et al. 2005; Lo Vecchio 2015).

4.7 Experimental and clinical implications and limitations

The UVB inflammatory model induces a well-defined non-neurogenic inflammatory reaction accompanied by erythema, increased cutaneous blood flow, and pigmentation. This model was well tolerated and the only reported side effect was a hyperpigmentation of the irradiated skin lasting several weeks. The 24 h capsaicin-desensitization model was well tolerated by the included participants despite the discomfort and the pain associated with its administration. The most affected parameters were those related to thermal sensitivity. Still, the magnitude of decrease in thermal sensitivity present in this study does not cause any safety concern, moreover there are no clinical studies indicating capsaicin-induced disruption of protective thermal sensation (Forst et al. 2002; Malmberg et al. 2004). The most important limitation of this study was linked to the difficulty of blinding the test participants with regard to the vehicle/capsaicin treatment due to the mild-to-moderate pain induced by capsaicin during application. Blinding was attempted by randomizing the capsaicin site of application so that the two capsaicin patches were always placed in different forearms. Moreover, in order to further blind the subject to the active treatment (capsaicin vs vehicle), the patches were covered with opaque medical tape. Another aspect that should be taken into consideration is related to the participant expectation. Even though the test subjects were unaware of the effects of capsaicin, the long-time application could have cause some expectation that could have affected the results. Even if this event is unlikely, it should be considered in further studies. However, the 24 h capsaicin-ablation model can be safely used to study cutaneous afferents and to test new TRPV1 antagonists.

5 Conclusion

The thermal hyperalgesia induced by UVB inflammatory model was prevented in capsaicin-treated skin while the mechanical hyperalgesia was only partially impaired after capsaicin ablation. The 8% capsaicin-ablation method predominantly defunctionalizes $TRPV1^+$-expressing cutaneous nociceptors while does only partially affect mechanical pain detection. The data suggest that humans $TRPV1^+$-expressing fibers are involved in the development of heat hyperalgesia following cutaneous UVB inflammation, but they are partially necessary for the establishment of robust primary mechanical hyperalgesia.

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Figure Legend

Figure 1 Study design. Flowchart of the study design, sensory testing and vasomotor assessments performed in the 4 areas and randomization of UVB irradiation and capsaicin patches to the volar forearms

Figure 2 Pain intensity after the 8% capsaicin patches application. **A)** 6 hours pain score in the left and right forearm; **B)** Average of rated pain intensity and peak pain during the 24 h patches application. Mean ± SEM shown. No differences (≠) were present between left and right arm.

Figure 3 Sensory changes caused by the 8% capsaicin and the UVB irradiation. **A**) Warmth detection threshold, **B**) Heat pain threshold, **C**) Supra-threshold heat sensitivity, **D**) Mechanical pain threshold, **E**) Mechanical pain sensitivity, **F**) Touch Pleasantness. The asterisk marks represent significant differences between the active treatment and the vehicle condition; * P<0.05, ** P<0.01 and *** P<0.001. The hash marks represent significant differences between the two capsaicin areas; # P<0.05, ### P<0.001. The dot marks represent significant differences between the two UVB areas; • P<0.05, •• P< 0.01, ••• P<0.001

Figure 4. Sensory changes caused by the 8% capsaicin and the UVB irradiation. Each plot represents a sensory test and shows the Δ-value calculate by subtracting the result from the placebo-treated area within each session with the active treatments. **A**) Warmth detection threshold, **B**) Heat pain threshold, **C**) Supra-threshold heat sensitivity, **D**) Mechanical pain threshold, **E**) Mechanical pain sensitivity, **F**) Touch Pleasantness. The asterisk marks represent significant differences between the active treatment and the vehicle condition. * P<0.05, ** P<0.01 and *** P<0.001. The hash marks represent significant differences between the two capsaicin areas. # P<0.05, ### P<0.001. The dot marks represent significant differences between the two UVB areas. • P<0.05, •• P< 0.01, ••• P<0.001

Figure 5 Changes in erythema, pigmentation, water loss and neurogenic response induced by the 8% capsaicin and the UVB irradiation. **A**) Erythema, **B**) Skin pigmentation, **C**) Trans-epidermal water loss, **D**) Mean superficial perfusion.

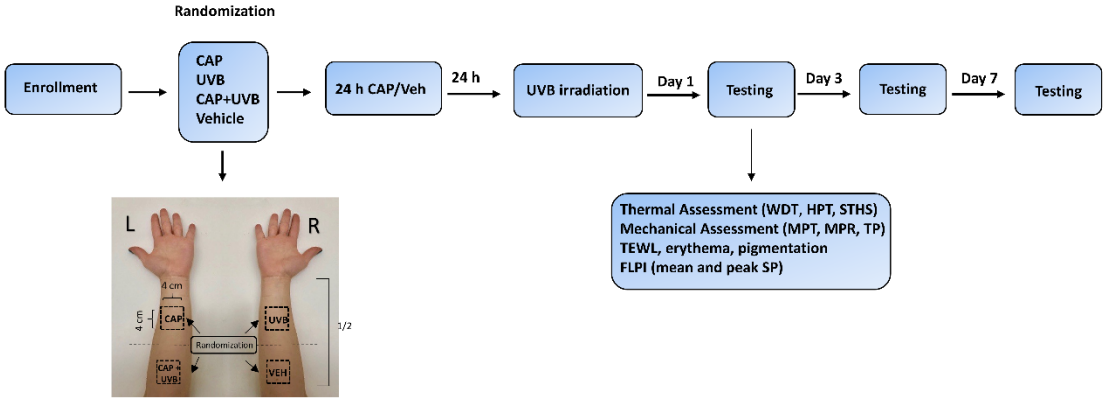

Figure 1

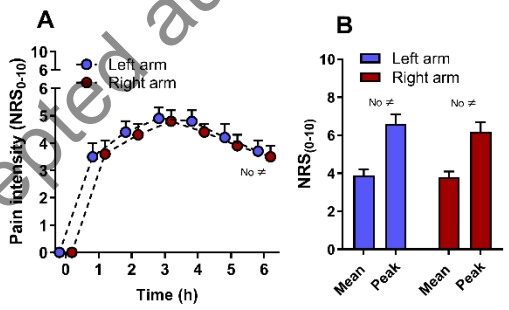

Figure 2

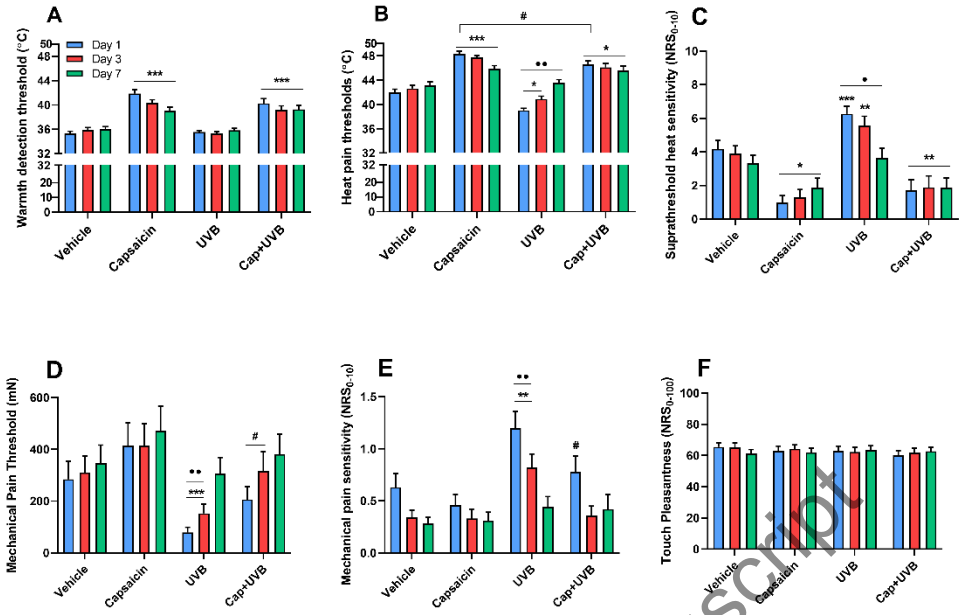

Figure 3

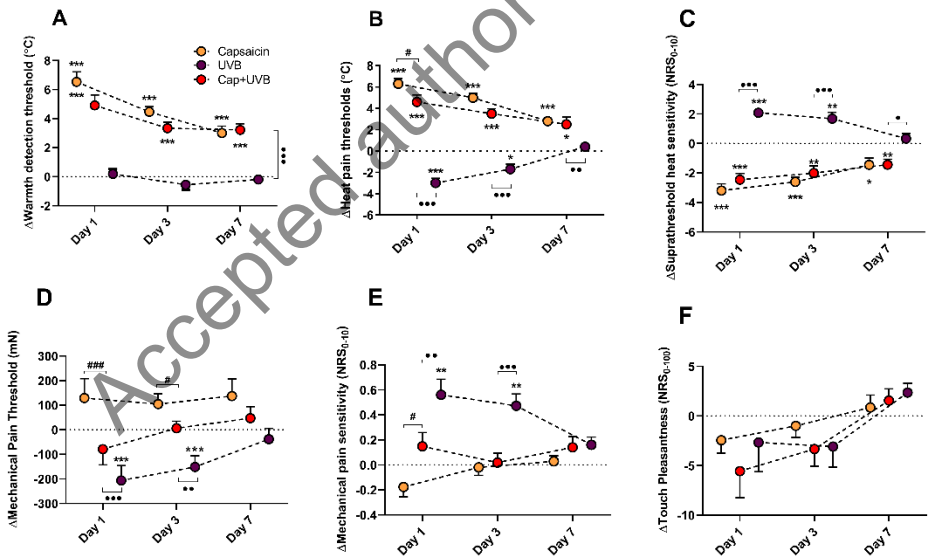

Figure 4

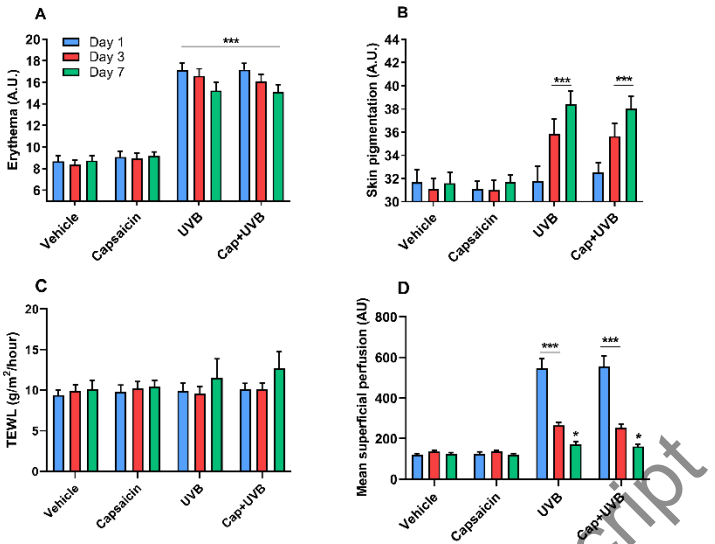

Figure 5