APPENDIX S1

SUPPLEMENTARY METHODS

This study included a total of 19 healthy subjects (23 years ± 3.31; M12, F7). Exclusion criteria were pregnancy and lactation, ongoing pain, medication, or drug use, previous or current mental, neurological, dermatological, immunological, musculoskeletal, or skin diseases. All subjects signed an informed consent, and the regional ethics committee approved the protocol (N-20200005).

Each volar forearm of the subject was divided into two squared areas (4x4 cm), 4 cm apart. Each area was localized at exactly 2 cm from the center of the volar forearm, one toward the wrist and the other towards the elbow. In the 1st session, the areas were randomly exposed to different concentrations of papain (Papain from papaya latex lyophilized powder, ≥10 units/mg protein, Sigma-Aldrich/Merck, Denmark; 10, 50, 100 µg in 20 µl of papain in distilled water, corresponding to final concentration of 0.5, 2,5 and 5 mg/ml) and a vehicle through a single standard skin prick test (SPT), by using a weighted custom-made 120 g SPT device. The 10 µg and 100 µg papain was previously used by Simone et al. (14). In the 2nd session (7 days later), the areas were randomly exposed to 5mg/ml papain delivered through different amounts of SPT pricks (1, 5, and 25 SPT pricks), and heat-inactivated (by autoclaving for 50 min at 121°C) cowage spicules soaked overnight in the same papain solution. The inactivated spicules were inserted into the skin by gently rubbing in a circular motion, and removed after 15 min by using a tape. The concentration of 5 mg/ml corresponds to the highest among the three used, and it has been chosen to ensure that a moderate amount of itch could be induced. Then, pain and itch were monitored for 15 minutes after each application by a visual analog scale (VAS) ranging from 0 (= "no itch"/"no pain") to 100 (= "worst imaginable itch"/" worst imaginable pain"). Immediately after, measurements of superficial blood perfusion with full-field laser perfusion imaging (FLPI2, Moor Instruments, Axminster, Devon, England) were conducted, followed by measurements of touch pleasantness (TP), mechanically evoked itch (MEI), mechanical pain thresholds (MPT) and sensitivity (MPS), and lastly thermal assessments. Measurement of TP was conducted using a standardized sensory brush (SENSELab Brush-05, Somedic AB, Hörby, Sweden) exerting a force of 200 to 400 mN and rated through a numerical rating scale $NRS_{0\text{-}10}$ labeled "very unpleasant" and "very pleasant" at the extremity and "neutral" at the center. MEI was assessed by applying 3x3 stimulations with von Frey filaments (1.0 g, 1.4 g, and 2.0 g), rated using $NRS_{0–10}$ scale where 0= "no itch", and 10=" worst imaginable itch". MPT and MPS were assessed using a pin-prick set (Aalborg University, Aalborg) of 7 pins with diameters of 0.6 mm and different force applications (8 mN, 16 mN, 32 mN, 64 mN, 128 mN, 256 mN, and 512 mN). The assessment of MPT started with the lightest stimulator and continued in ascending order until the first perception of sharpness was felt. The final threshold was the geometric mean of five series of ascending and descending stimuli (method of limits). MPS was conducted twice in an ascending application order, where each stimulus was rated on an $NRS_{0–10}$ scale (0 indicated ''no pain'', and 10 indicated the ''worst imaginable pain''). Thermal assessments were conducted

by a PATHWAY ATS (Medoc Ltd, Israel) thermal sensory testing device with a thermode stimulator of 3x3 cm. The starting temperature was 32°C and increased or decreased by 1°C per second until the subject terminated the measurement depending on whether cold (CDT) and warm detection thresholds (WDT) or pain thresholds (CPT and HPT) were assessed. For measurement of two supra-threshold heat stimuli (STHS), the subjects had to rate the pain from 0 (= ”no pain”) to 10 (= ”worst imaginable pain”) starting and ending at 32°C with an increase and decrease of 5°C/s and 3 seconds plateau at 50°C. A cut-off temperature of 0°C or 50°C was used in all the tests.

The sample size was calculated in G*Power 3.1.9.4, Universität Düsseldorf. For the calculation, type I error was set to 5% (alpha = 0.05), type II error was set to 20% (80% power), the effect size was set to 0.3 (small-to-moderate effect), and the intercorrelation was estimated to 0.5. Statistical testing was performed in SPSS. Statistical tests included repeated measures of ANOVA using Sidak post hoc tests with *condition* (8 levels indicating the vehicle, 10 µg papain, 50 µg papain, 100 µg papain, 1 prick, 5 pricks, 25 pricks, and inactivated spicules) as the only factor. For non-normally distributed data, Friedman Test was performed following Wilcoxon Signed Rank tests with Bonferroni correction. A significance level of p-value ≤ 0.05 was used. The graph plotting was made using GraphPad Prism 9 (GraphPad Software Inc., CA, USA).

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