Acute effect of different concentrations of cayenne pepper cataplasm on sensory-motor functions and serum levels of inflammation-related biomarkers in healthy subjects

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Abstract

Physical medicine therapies are often used in treating widespread musculoskeletal disorders, such as neck and low back pain. Herbal cataplasms containing rubefacient substances, such as Cayenne pepper, or galenic preparations like Munari cataplasm are commonly used as natural medications to treat painful areas. In this paper we show the effects of a 20-min application of Cayenne pepper and kaolin powder cataplasm (CPC) on healthy subjects. Treatment effects were evaluated by cold/hot feeling on visual analogue scale, blood pressure, body temperature, skin light touch sensations, two-point discrimination, and pain threshold to a mechanical stimulus, before and immediately after, 15 min after and 30 min after different concentration of Cayenne pepper in CPC preparation on healthy subjects. Maximal voluntary trunk extension force and trunk extension submaximal force matching error were also measured. In addition, the resulting optimal CPC mixture was tested for its safety by measuring changes in circulating levels of inflammatory-related biomarkers after 20-min application. The results indicate that the 5% concentration of Cayenne pepper in the preparation of CPC is the best choice, since no additional effects can be obtained with the 10% concentration, and the effects are higher than those observed at the 2.5% concentration. Importantly, 5% CPC application did not induce a significant increase of inflammatory-related biomarkers, suggesting that 20-min application has no negative side effects at systemic levels. Further studies are needed to investigate the immediate and long-term effects of repeated CPC applications as well as to understand the intersecting underlying mechanisms activated by Capsaicin and other identified factors, in order to be more extensively used in the field of physical medicine therapies.

Key Words: Capsicum, capsaicin, TRPV1 receptor, physical therapy modalities, pain, sensation, inflammation, IL-6.

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Musculoskeletal diseases, such as neck and low back pain, are widespread disorders in many developed countries. Their management is challenging, and it may have mounting socioeconomic burden.[1] Several evidences in the literature demonstrate the efficacy of many therapeutic strategies in the treatment of these conditions, based on pharmacological or surgical interventions.[2-6] Physical medicine therapies are good alternatives that may have beneficial effects, especially when used as first line of intervention before approaching more expensive pharmacological or invasive medications[7-9] The beneficial analgesic effects of *Applicatio Epispasticorum* of *Rubefacenciae* (redden skin substances), is known since 1830 as a treatment for pain, cramps, and disorders of the musculoskeletal system.[10] Herbal cataplasms containing rubefacient substances, such as Cayenne pepper (CP), are commonly used as natural medications to treat painful or aching areas in the case of acute or chronic back pain, and

Table 1. *Terminology used through the study to describe CPC preparation and application.*

Term	Synonyms	Description
CP powder		Dry mixture of Cayenne pepper and Kaolin powders
CP cataplasm (CPC)	CP poultice	Soft heated mass of Cayenne powder melted in water
CP patches		CP poultice spreads on a sheet of paper
CP application	CP treatment	CP patches applied to the skin

rheumatisms.[11] They are also used in pain involving joints caused by osteoarthritis. A galenic preparation composed of rubefacient substances of vegetable origin, which generated vasodilation and increase in blood circulation on the treated areas, was first prescribed in 1909 by Dr. Giuseppe Munari to treat pain of various areas of the locomotive system. He proposed a method based on applications prepared according to his own galenic formula that have become famous all over Italy and Europe. In Vienna and lower Austria, a Munari-like application containing CP and Kaolin powder in mixed proportion, is commonly used to treat musculoskeletal conditions of pain especially of the low back.[12] In CP, more than 20 capsaicinoids are present such as capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin.[13] They are alkaloid compounds that are synthesized and accumulate in pepper fruit, having irritant properties. Capsaicin is the most abundant capsaicinoid present in the CP1,[14] and it is an agonist of Transient Receptor Potential Vanilloid 1 (TRPV1), a polymodal transmembrane receptor found on cutaneous and muscle nociceptive sensory nerves (C and Aδ fibers) which integrates a number of painful stimuli including heat, extracellular acidification, and inflammatory mediators.[15] This receptor is a nonselective cation channel that is highly permeable to calcium.[16] Cataplasm containing CP (CPC) directly applied to the skin at the site of the painful areas, provokes a hyperemic response, that involves both epidermis and muscle tissue nociceptor fibers, with beneficial analgesic effects. Usually the application is carried out for 20 min, two to three times a week, for a total of 10 applications. This treatment is generally well tolerated, but no detailed information about the galenic preparation, in particular related to CP concentration in the powder, are available, and data about the potential side effects and secondary targets of this treatment are missing.

The aims of the present study were to test on healthy subjects i) the optimal concentration of CP for cataplasm preparation assessing its efficacy on selected functional and mobility parameters compared to placebo; ii) safety of the treatment by testing the effects of the resulting optimal CPC preparation, on circulating biomarkers of inflammation, blood vessels activation, and neuroendocrine stress.

Materials and Methods

Participants

In the Concentration study, twenty healthy adults (14 females,. 6 males; age 43.2 ± 4.6 years, body height 170.2 ± 81 cm, body mass 75.7 ± 19.3 kg) were included. The sample size was calculated based on pilot data ($p < .05$, $\beta = .20$). Inclusion criteria (self-reported) were: 30 to 50 years of age, body mass index below 30 kg/m2,

Table 2. *Total capsaicin concentration in CP was 902mg/kg = 0.09% (analyzed by Eurofins Analytik GmbH, Wiertz-Eggert-Jörissen, Neuländer Kamp 1, Hamburg, Germany)*

Kaolin [%]	Cayenne Pepper [%]	Capsaicin content in Cayenne powder [%]
100	0.0	0.00000
97.5	2.5	0.00225
95.0	5.0	0.00450
90.0	10.0	0.00900

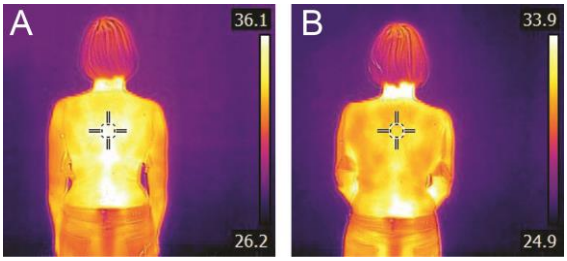

Fig 1. *A representative subject`s thermographic scan of the trunk before (A) and immediately after (B) the 20-min CPC application containing 5% of CP powder in one of the subjects included in the Safety Study. Note the difference between the min/max values on the colour coded scale*

moderately physically active (sport participation, mid-to-high intensity, 2-3 x/week, ≥ 30 min/session), absence of any health problems for the last three months. Exclusion criteria were: current or recent (< 6 months) pain in lumbopelvic region and smoking. In the Safety study, six healthy adults (6 males, 51.65 ± 8.79 years, body height 176,50 ± 170.2 cm, body mass 80.50 ± 10.3 kg) were recruited. Inclusion criteria (self-reported) were: medical doctors, 35 to 65 years of age, body mass index below 30 kg/m2, moderately physically active (sport participation, mid-to-high intensity, 2-3 x/week, ≥ 30 min/session), absence of any health problems for the last three months. Exclusion criteria were: current or recent (< 6 months) pain in lumbopelvic region.

Experimental design

Concentration study: Each participant underwent four applications of CPC (see Table 1 for terminology) with 7 to 10 days in between. The procedure during each visit was the same: (1) before-treatment assessments, (2) a 20-min application of CP patches to the back of the body, and (3) after treatment assessment(s) after the removal of the patches. On each visit, different concentration of CP in the powder (0% (i.e. placebo), 2.5%, 5%, and 10%, see Table 2) was used to prepare the cataplasm in random order. Safety study: Each participant underwent one application of 5% CP patches for 20-min. Blood samples for serum isolation (7.5 ml) were collected before and after (post- 0.5 hr, 1hr, 3 hr, 6 hr, and 24 hr) CPC application.All subjects were not fasted at the time of blood sampling. Serum was separated by centrifugation carried out at 3000 rpm for 10 min, and stored in 500 µl aliquots at −80 °C until assays.

Preparation of CP poultice and patches

Two CP patches were prepared according to the below described standard procedure. A pre-prepared concentration of CP powder (Table 2) was added into cold water by spoon, and mixed until the poultice reached smooth structure and heated to 60-70°C using a steam pot. The CPC was spread on a thin sheet of paper (37 cm

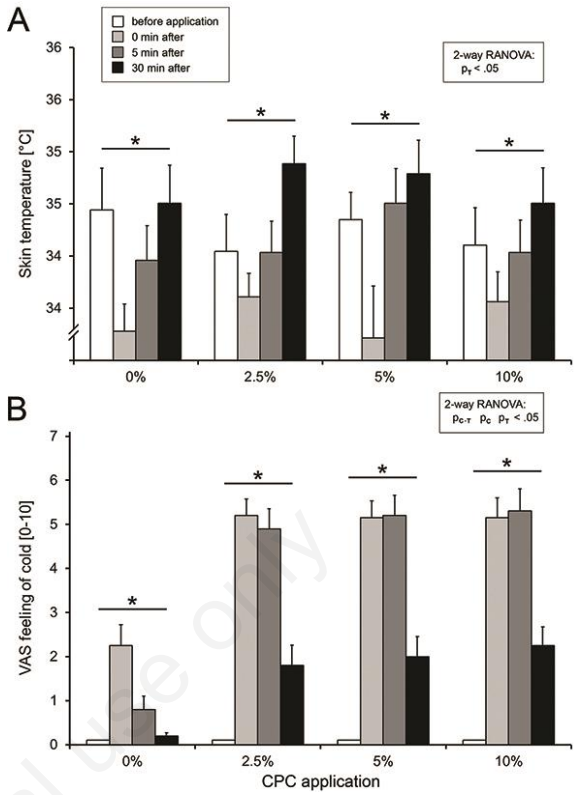

Fig 2. *Changes of skin temperature (A) and change of cold feeling (B) in the area of cataplasm application (0, 2.5, 5.0, and 10 % concentrations of CP powder). In the top-right of each graph statistically significant outcomes of 2-way repeated measures ANOVA are indicated (pC, pT, and pC·T = statistical significance for factor Concentration, Time, and Concentration × Time interaction, respectively. The asterisks indicate statistically significant differences for 1-way repeated measures ANOVA (p < .05).*

x 25 cm) and used as a patch for application. Two to three patches, depending on the subject`s trunk size, were placed directly on the skin (45-50°C), spanning over the whole back - from C7 down to the S1 level. The patches were then covered with a towel, on top of which two 0.5 kg sand bags were placed to assure for a good contact. Room temperature was set to 24°C.

Measurement procedures and serological tests

Concentration study

Sensation of cold by the subject during CPC application was tested using a standard 0-10 visual analogue scale (0 = absence of cold sensation; 10 = maximum feeling of cold). Skin temperature was measured at L3 level 3 cm laterally from midline using a thermographic camera (E6, FLIR Systems, Wilsonville, USA), from a 2-m distance. Blood pressure was measured using an automatic

Table 3. *Serum levels of soluble cell adhesion molecules before and after CPC treatment*

	sVCAM (pg/ml)	sp-selectin (ng/ml)
Reference value	**584,58 pg/ml**	**41,62 pg/ml**
Pre	597,75±151,80	30,59±11,71
0.5 hr post	604,80±134,80	29,24±8,59
1 hr post	614,02±148,50	29,79±9,20
3 hr post	582,12±153,16	28,23±9,39
6 hr post	531,62±169,73	25,38±3,70
24 hr post	520,65±146,36	28,44±5,34

sVCAM-1= soluble vascular cell adhesion molecule 1; sp-selectin= soluble platelet selectin.
Values are expressed as mean ± SD.

barometer (Omron Healthcare, Netherlands) with the cuff placed around the right upper arm. The subject was seated on a chair with the hands relaxed and supported on the thighs. Sensitivity to light touch was tested in a laying position using monofilaments calibrated to 0.025, 0.07, 2, 5, and 10 grams (West D, FBC-121408, Health Mega Mall, Bellingham, USA). The subject was asked to report when he/she felt touching the skin with a monofilament 3 cm laterally from L3 spinal process. The skin was touched 3 times with each monofilament, starting with the thinnest and progressing (~1 s touch separated by ~2 s). The monofilament thickness at which the subject felt at least 2 out of 3 touches, was marked as the final result. Two-point discrimination test was carried out using two pins comparable to a two-point discriminator (FBC-121495, Health Mega Mall, Bellingham, USA). The pins were oriented in medial-lateral direction and pressed perpendicularly to the skin, with the medial pin being 3 cm laterally from the L3 spinal process5. The subject was asked to report whether he/she felt one or two pins. Each inter-pin distance was used three times using single pin touches in between for a control. The smallest inter-pin distance which the subject reported correctly at least 2 out of 3 repetitions was taken as the result of the test. The threshold for pain sensation was tested using a mechanical algometer (9024.80, Wagner Instruments, Greenwich, USA). The subject was lying prone and the algometer tip was applied at the same site as the monofilaments and perpendicularly pressed against the body progressively (~25 kg/s), until the subject reported to feel pain. Three repetitions, separated by ~10 s were carried out and the average value was used for further statistical analysis. Maximal voluntary trunk extension force was tested using an isometric dynamometer (S2P Ltd., Ljubljana, Slovenia). The subject adopted an upright standing position, with the upper edge of the support bar placed on the level of anterior superior iliac spine and a rigid strap fastened across the pelvic girdle. The subject was instructed to perform three maximal voluntary isometric trunk extensions, separated by 20-s intervals. A single muscle action gradually increased over ~2 s, followed by ~3 s of maximal effort. The subject was verbally encouraged. The highest mean force output within one-s time interval was included into the further statistical analysis. This maximal voluntary contraction testing protocol has been used in the past.[17] Trunk extension submaximal force matching error was tested using the same isometric dynamometer and the same positioning. The subject was asked to: (1) develop submaximal force of 30 % maximum based on visual feedback and sustain it for ~2 s, (2) relax for 2 s, and (3) develop the same amount of the submaximal force again without any feedback. Three pairs of submaximal contractions were repeated with a 20-s rest intervals in-between. A force matching error was calculated as a difference between average force on the middle 1-s interval of the repetitions. Mean value calculated from the three task repetitions was included into the further statistical analysis. Trunk position sense was tested as a repositioning error for the trunk forward bent task. The test was performed in a standing position and with vision restricted. The subject slowly bent the trunk forwards and was stopped verbally within the middle 50% of his/her range of motion. This position was held for ~2s. Then the subject returned back to the upright posture and repeated the task by him/herself three times. The end position of the trunk forward bend was measured using a digital inclinometer (Baseline, model 12-1057), positioned with a caudal pin at promontory. The test was repeated three times. The outcome result of each pair of repetitions was the absolute difference in angles. The three repetitions were averaged and used for further statistical analysis.

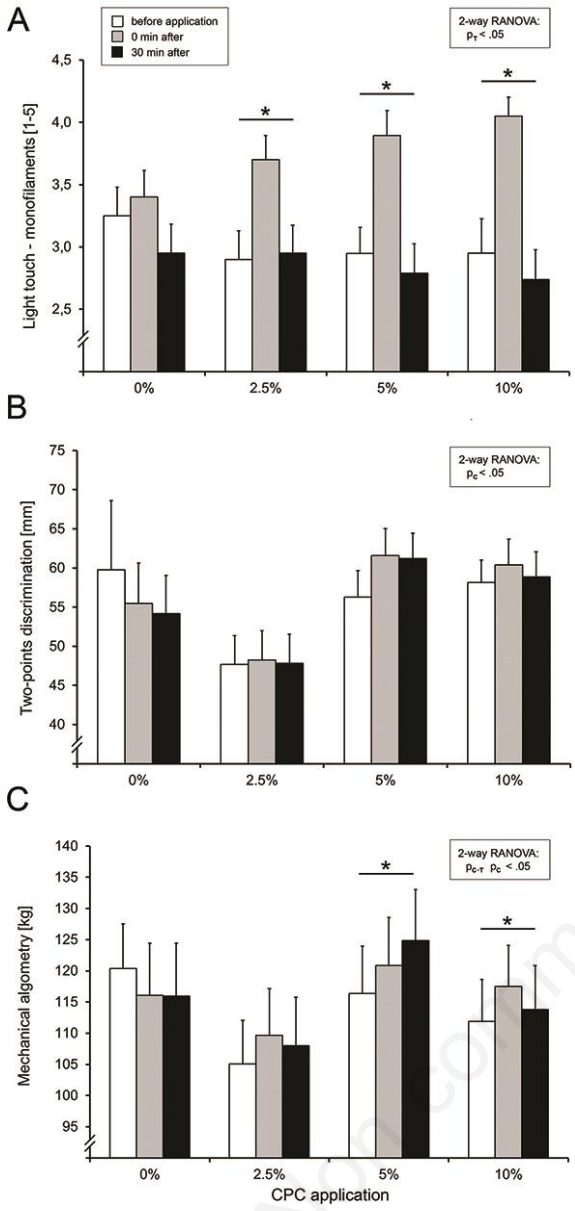

Fig 3. *Changes of thresholds for: light touch perception (A), discrimination of two points/pins (B), and perception of pain to mechanical stimulus (C) for 0, 2.5, 5.0, and 10 % concentrations of CP powder. In the top-right of each graph statistically significant outcomes of 2-way repeated measures ANOVA are indicated (pC, pT, and pC·T = statistical significance for factor Concentration, Time, and Concentration × Time interaction, respectively. The asterisks indicate statistically significant differences for 1-way repeated measures ANOVA (p < .05).*

Safety study

Systemic levels of inflammatory and endothelial cell adhesion molecules.

Tumor Necrosis Factor alpha (TNF-α) (Human TNF-α Immunoassay HSTA00D), Interleukin 1 beta (IL-1β) (Human IL-1β/IL-1F2 Immunoassay HSLB00C), Human TGF-β1 (Human TGF-β1 Immunoassay DB100B), and Interleukin 6 (IL-6) (Human IL-6 Immunoassay HS600B), and the soluble form of cell adhesion molecules Vascular Cell Adhesion Molecule-1 (sVCAM-1) (Human sVCAM-1/CD106 Immunoassay DVC00), sP-Selectin (Human sP-Selectin/CD62P Immunoassay BBE6), sE-selectin (Human sE-Selectin/CD62E Immunoassay DSLE00), were measured by ELISA following manufacture's instructions. All kits were purchased from R&D Systems Inc., Abingdon, U. To avoid inter-assay variations, all samples were analysed with the same kit on the same day.

Serum detection of acute phase proteins and neuroendocrine stress hormone

C-reactive protein, and albumin, were analyzed on a Siemens Dimension Vista 1500 multiparametric analyzer (Siemens Healthcare Diagnostics, Erlangen, Germany); cortisol was analyzed on a Siemens Centaur XP Immunoassay System. The Erythrocyte sendtimentation rate (ESR) was measured with an Alifax THL sedimentation analyzer (SIRE Analytical Systems, Nimis, Italy).

Statistical analyses

For each of the measured parameters, means, standard deviations and were calculated across subjects. Shapiro-Wilk test was used to test for normality of the distribution. Differences in the measured variables between the time points (before vs. after CPC application) and CPC (i.e. capsaicin) concentrations were analyzed with a 2-way repeated measures analysis of variance (RANOVA) (Concentration (2) x Time (2 or 4)). When 2-way RANOVA was found significant, 1-way RANOVA was performed for each CPC concentration separately. All statistical analyses were performed using the IBM SPSS statistics 21.0 software (Armonk, NY, USA). Concentration of serological biomarkers were expressed as mean ± standard deviations (SD) and standard error of the mean (SEM). Differences in circulating levels before and after application at the indicated time points were analyzed with Student *t* test and 1-way ANOVA using the GraphPad Prism 5.03 software (La Jolla, CA, USA). The level of statistical significance for all tests was set at $p < 0.05$.

Results

Concentration study: CP concentration effects on pain, sensory functions, range of motion and maximal voluntary force .

For the skin temperature, a systematic *Time* effect was found ($F = 12.650$, $p = .000$, $ES = .442$), while the *Concentration* and the interaction (*Time* × *Concentration*) effects were not statistically significant ($F = 0.238$, $p = .869$, $ES = .015$ and $F = 1.160$, $p = .325$,

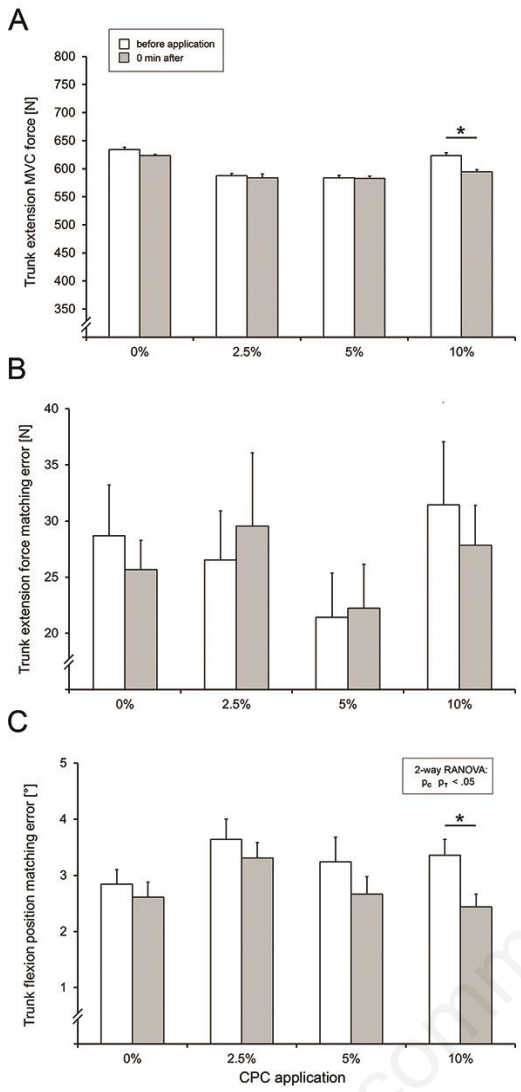

Fig 4. *Changes from before to immediately after the 20-min cataplasm application for: maximal voluntary trunk isometric force (A), submaximal trunk extension isometric force matching error (B), and trunk flexion, i.e. forward bending, position matching error (C); for 0, 2.5, 5.0, and 10 % concentrations of CP powder. In the top-right of each graph statistically significant outcomes of 2-way repeated measures ANOVA are indicated (pC, pT, and pC·T = statistical significance for factor Concentration, Time, and Concentration × Time interaction, respectively. The asterisks indicate statistically significant differences for 1-way repeated measures ANOVA (p < .05).*

ES = .068, respectively) (Figure 2A). Additionally, one-way RANOVA showed that this statistically significant effect was present for all the capsaicin, including the 0% (i.e. placebo) (F = 5.554 – 14.611, p = .000 – 0.007, ES = .211 - .448). *Post hoc* tests revealed the most intensive change in skin temperature immediately after the CPC application, with return to initial values after 15 min (before vs. 15 min after: p > .05). A tendency of an increased skin temperature was observed at 30 min after the CPC application, but not after placebo application (before vs. 30 min after: p = .054, .013, and .149 for 2.5%, 5.0%, and 10% concentrations, respectively). Neither systolic nor diastolic blood pressure changed significantly in any of the used CPC concentrations (p > .05). Subjective perception of cold (Figure 2B) only partly coincided with the actual skin temperature. Statistically significant differences were observed for both factors separately (*Concentration*: F = 45.254, p = .000, ES = .715 and *Time*: F = 68.408, p = .000, ES = .792) as well as for their interaction (F = 10.159, p = .000, ES = .361). In case of cataplasm applications containing CP the subjects graded their feeling of cold on average ~5 immediately after and 15 min after the application (all p < .032). In these cases, the perception of cold halved at 30 min after the removal of the CPC application. Although the described kinetic of cold sensation was similar for all the four conditions, the immediate average changes in placebo were almost three times smaller (p < .05), in respect to applications containing CP, with no significant carry over effect (before vs. 30 min after: p > .05). Comparison of the somatosensory perception results before vs. immediately after the CPC application showed: (1) decreased sensitivity to light touch for all concentrations except placebo, (2) no changes in two-point discrimination minimal distance, and (3) decreased sensitivity to pain evoked by mechanical pressure in case of 5% and 10% CP concentrations (Figure 3A-C). Taking all the three observed time points together (before, after 0 min and after 30 min), a significant *Time* effect was observed for the results of the monofilament test (F = 45.035, p = .000, ES = .726). This decrease in light touch perception threshold was comparable between 5% and 10% CP concentrations (F = 11.591 and 12.081, p = .000, ES = .392 and .402, respectively) but less pronounced when the 2.5% concentration was applied (F = 4.724, p = .015, ES = .199). Results of the pain threshold, however, showed no across conditions *Time* effect (p > .05), but differences among different CP concentrations were present as represented by a statistically significant *Time* × *Concentration* effect (F = 2.667, p = .019, ES = .136). *Post hoc* analysis of the algometry outcomes revealed that no significant changes took place when a 0% or 2.5% concentrations were used (p >> .05, ES < .07), but, the pain threshold statistically significantly increased when a cataplasm of either 5% or 10% CP concentration was applied (F = 3.364 and 3.388, p = .049 and .047, ES = .160 and .134, respectively). Although the two-point discrimination main effects were not observed (p > .05), *post hoc* tests showed some tendency of inter-pin distance decrease immediately after the 20-min application in case of 5% and 10% concentrations (p = .093 and .049 respectively). Results for the maximal

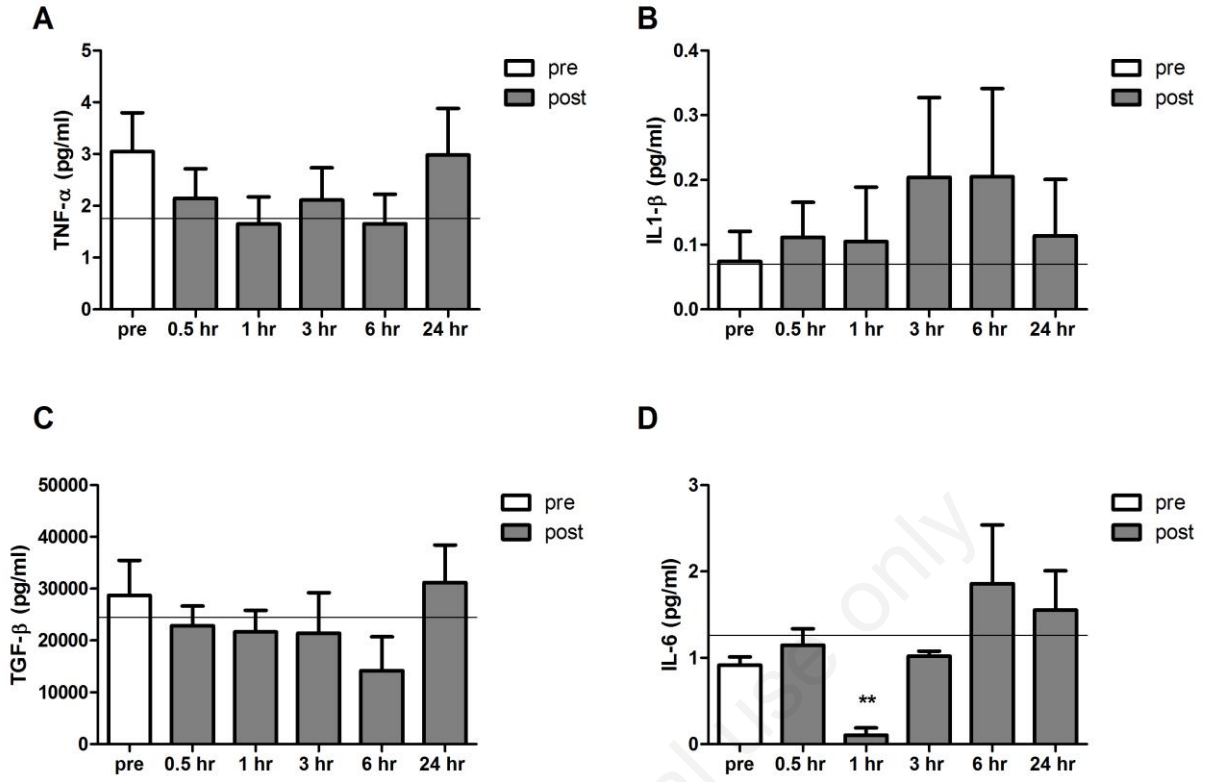

Fig 5. *Serum concentration of inflammatory cytokines TNF-α (a), IL-1β (b), TGF-β (c), and IL-6 (d) in healthy subjects before (pre-treatment) and after (post- 0.5 hr, 1hr, 3 hr, 6 hr, and 24 hr) 20min 5% CPC application. Columns represent the mean ± SEM of independent assays performed in duplicate for each subject. **p < 0.01; with respect to pre values. The horizontal line represents the mean serum concentration, as a reference value, determined in the laboratory in healthy people of the same age range A) TNF-α 1,76 pg/ ml; B)IL-1β 0.07 pg/ml; C) TGF-β 24.455,27 pg/mL; D) IL-6 1.26 pg/mL).*

voluntary trunk extension isometric force and both matching tasks (submaximal isometric force and trunk flexion position) are presented in Figure 4. The main *Time* effect for maximal voluntary force was not statistically significant (F = 7.311, p = .142, ES = .122), however, a significant drop in voluntary force was observed in 10% CP concentration (F = 14.568, p = .001, ES = .434). No statistically significant changes were found for the submaximal force matching error, neither using 2-way nor using 1-way RANOVA (all p > .40, all ES < .039). Trunk re-positioning error was increased after the 10% CP application (F = 6.909, p = .017, ES = .267). This coincided with a significant main *Time* effect (F = 7.311, p = .015, ES = .289) for the re-positioning error.

Safety study: 5% CPC effects at site of application and on systemic levels of inflammatory and cell adhesion molecules

The skin effect of 20-min CPC application containing 5% of CP powder is shown in Figure 1. Comparing the thermographic scan of the trunk before (A) and immediately after (B) the treatment, difference between the min/max values on the colour coded scale can be observed, which are indicative of a decrease in body temperature at the site of application. Consistent with that, all subjects felt warm/burning and tingling on the back during and after 20-min application, even though with different intensities. Figure 5 shows the effects of a single application of 5% CPC on circulating cytokines. Serum concentrations of the pro-inflammatory cytokines TNF-α (Fig. 5 A), IL-1β (Fig. 5 B), and the anti-inflammatory cytokine TGF-β (Fig. 5 C) changed over time, even though not significantly, returning to the basal levels within 24h after the treatment. The concentration of the regulatory cytokine IL-6 (Fig. 5 D) was significantly reduced, in all analyzed samples, 1h after the treatment, but increased immediately after and reaching the basal values at 24h. Serum concentration of CRP and Albumin did not significantly change in response to the treatment (Figure 6, A and B). CRP levels were exactly the same in all subjects and no changes were observed after 5% CPC application. A fluctuation in the serum levels of Albumin was observed across analyzed

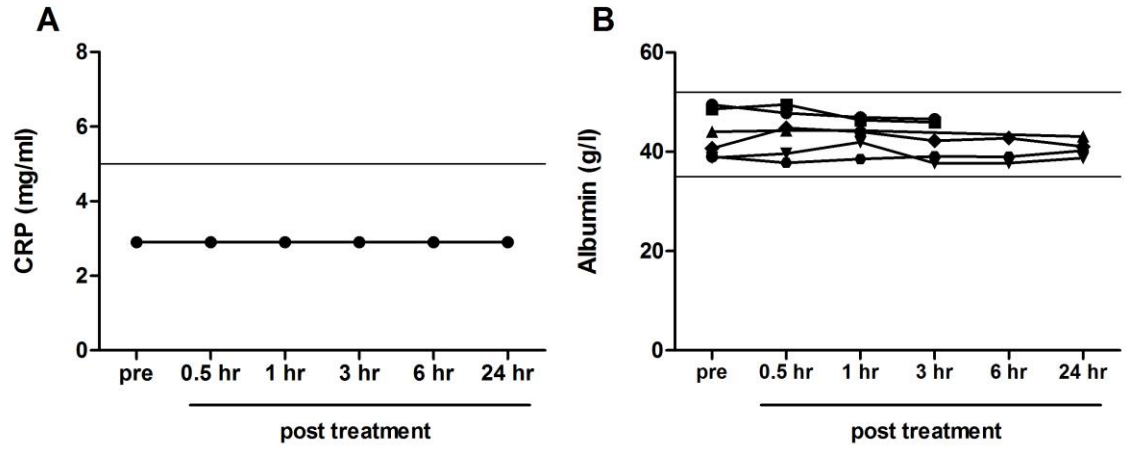

Fig 6. *Serum concentration of acute phase proteins C Reactive Protein (CRP) and Albumin in healthy subjects before (pre- treatment) and after (post- 0.5 hr, 1hr, 3 hr, 6 hr, and 24 hr) 20min of 5% CPC application. Dots represent the mean value of technical duplicate for each participant at each time point. Different symbol shape represents a different subject. The horizontal lines indicate the upper and lower reference values.*

time period, with differences between the subjects, even though not significant. All values were detected within the normal reference range. Similarly, serum levels of markers related to the activation or damage of platelets fluctuate in response to treatment, but without significant variation, and their concentration were below the reference values (Table 3). The soluble form of endothelial cell adhesion molecule was significantly reduced after the treatment in comparison to the pre values, and did not return to the baseline levels after 24h (Figure 7). Cortisol serum levels tested before and after the treatment are represented in Figure 8. Treated subjects showed no significant variation in the systemic levels of this hormone, that was markedly reduced in particular at 6h after the treatment, but returning to the basal levels at 24h.

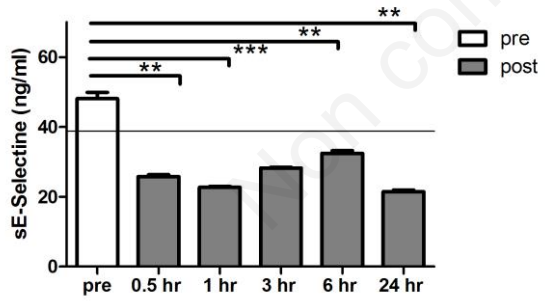

Fig 7. *Serum concentration of soluble Endothelial Selectin (sE-Selectin) in healthy subjects before (pre- treatment) and after (post- 0.5 hr, 1hr, 3 hr, 6 hr, and 24 hr) 20min of 5% CPC application. Columns represent the mean ± SEM of independent assays performed in duplicate for each subject. ***p < 0.0005; **p < 0.001; with respect to the pre values. The horizontal line represents the mean serum concentration, as a reference value, determined in the laboratory in healthy people of the same age. Value 38,77 ng/ml.*

Discussion

Creams containing capsaicin at low concentration for topical treatment of rheumatisms, arthritis, and musculoskeletal pain are commercially available (ABC Lokale Schmerz-Therapie - Waerme-Creme 750 µg/g

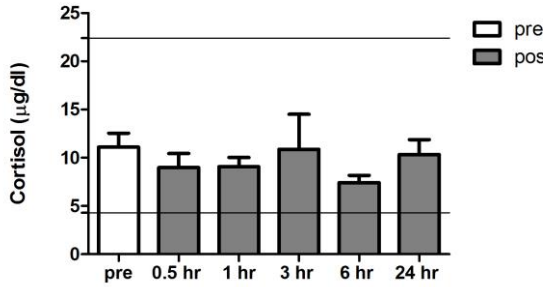

Fig 8. *Serum concentration of Cortisol in healthy subjects before (pre- treatment) and after (post- 0.5 hr, 1hr, 3 hr, 6 hr, and 24 hr) 20min of 5% CPC application. Columns represent the mean ± SEM of independent assays performed in duplicate for each subject. The horizontal lines represent the upper and lower reference values.*

Crème, Beiersdorf AG, Germann, ABC Lokale Schmerz-Therapie - Waerme-Pflaster Capsicum 11 mg, Beiersdorf AG, Germany, Trauma Salbe Mayrhofer wärmend, Kwizda Pharma GmbH, Austria) but multiple daily applications for a prolonged period of time are needed in order to achieve beneficial effects, thus increasing the cost of the treatment. Munari-like cataplasm (containing 0.0045% capsaicin in 5% CPC, Table 2) are commonly used as good alternative to creams having the additional advantage that it must be applied by physiotherapists experts in the field, controlling all the steps of the procedure, as a guarantee of the good outcome of the treatment.[18] Patches at high dose of capsaicin (8%) (Qutenza 179 mg kutanes Pflaster, GP Grenzach GMBH, Germany) are also commercially available and single applications of these patches are used for the treatment of acute and chronic neuropathic or post herpetic pain with good outcome[19,20], but they are not prescribed for rheumatisms, arthritis, and musculoskeletal pain. It has been documented that in 2013 only in the City of Vienna, 580.000 applications of Munari-like cataplasm containing CP were done (Friedrich Hartl, Bundesfachgruppenobmann für Physikalische Medizin und Allgemeine Rehabilitation, Österreichische Ärztekammer) showing a good outcome. Anyhow, at the best of our knowledge, there are no indications about the optimal concentration of CP to be used in cataplasm preparation or on the possible side effects at systemic levels of this preparation in healthy subjects.

Therefore, as first step of the present work, we tested three different concentrations of CP in the powder, to obtain further information about the optimal preparation resulting in significantly more pronounced and positive effects on the analyzed parameters investigating the immediate and carry over effects of a single CPC application in healthy subjects (*Concentration study*).

Our study demonstrate that a 20-min CPC application resulted in a significant drop of the lumbar back skin temperature, independently from CPC, the effect being visible also in the placebo. These findings may indicate that other factors (i.e. wet towels) can be responsible for the drop in skin temperature in the site of applications, that can partially counteract the vasodilation and hyperemia visible in those conditions in which capsaicin is added to the mixture. Anyhow, our results clearly show that CP (and therefore capsaicin contained in it) induces increase of skin temperature at 30 min after finishing the 20-min application of CPC at any tested concentration, while this phenomenon was not observed in the placebo. In line with these findings, subject perception of cold at the site of CPC application, coincided only partially with the measured skin temperature. On the other hand, the perception of cold in these subjects is linked to the drop of the temperature that can also be explained as a consequence of a possible perspiration at the site of application induced by hyperemia, as a mechanism activated in order to maintain the body temperature. Although the described kinetic of cold sensation was similar for all the four conditions, the immediate average changes in placebo were almost three times smaller ($p < .05$), in respect to applications containing CP, with no significant carry over effect (before vs. 30 min after: $p > .05$). This indicates that during CPC application several factors have vasomotor effects, but that Capsaicin is the crucial one in inducing hyperemia. Anyhow, it is well accepted that all the approaches of rehabilitation based on physical mechanisms have in common some hyperemia due to an acute transient inflammation, as an evidence of increased functional metabolism in the tissues accelerating the repair/regeneration mechanisms induced by the treatment. Clinical trials of Qutenza treatment (8% Capsaicin) reported elevated blood pressure in response to pain following applications of the patches[21]. Importantly, no effect on blood pressure were observed, as an indication that vasoconstriction and hyperemia are localized at the site of application, and do not alter the systemic blood pressure. For the great majority of the analyzed parameters, no additional statistically significant effect was observed with the 10% CP concentration compared to the 5% concentration. Noteworthy, 10% of CP concentration had effects on trunk extension MVC force and on trunk flexion repositioning error that significantly decreased immediately after a 20 min of CPC application. The MVC drop indicates a potential inhibitory effect on strength movement. On the other hand, a decrease of the trunk repositioning error means improvement of the kinesthetic function due to a potential sensitization of deep somatosensorics and conscious awareness of the treated body part. The effects in some of the measured parameters of sensory functions, in particular light touch skin sensation and sensitivity to pain evoked by pressure algometry, were stronger for 5% than for 2.5% CP concentrations. These effects were not observed in placebo, indicating that they are dependent on capsaicin-mediated activation of the known specific target, the TRPV1 receptors. When TRPV1 is activated by capsaicin or other external stimuli, it may open and initiate membrane depolarization mediated by the influx of sodium and calcium ions. Depolarization results in action potentials that propagate along the nerve fibers into the spinal cord and brain, resulting in burning sensations, hyperalgesia, allodynia, and erythema.[22,23] Furthermore, since TRPV1 is also expressed on intracellular organelles, such as endoplasmic reticulum and mitochondria,[24] external capsaicin application can cause release of calcium from the internal stores activating calcium dependent proteases that induce mitochondrial dysfunction, structural and functional changes in the fiber nerves,[24] These phenomena are responsible for the defunctionalization of the nociceptor fibers, resulting in sensory deprivation and consequent pain relief in suffering patients, when Capsaicin is used at high concentration or for prolonged period of time.[25]

Based on these evidences, that are indicative of both a dose-dependent effect possibly related to both the injury

of nociceptor fibers, and to increased perfusion in course of neurogenic inflammation, in the second part of the present work (*Safety study*), we aimed to test the possible side effects at systemic levels of the optimal CP application. We intended to do that although the concentration of Capsaicinin in our preparation was as low as 0.005%, because the treatment indeed induced local hyperemia, directly linked to vasodilation and inflammation. Locally produced mediators could have leaked from the vessels of treated skin, thus circulating systemically. We observed that 20-min treatment with 5% CPC, consisting of 0.0045 % of total Capsaicin, was sufficient to trigger an acute inflammatory response at the site of application, typically characterized by vasodilation and hyperemia, but did not induce a significant increase in the secretion of pro-inflammatory cytokines TNF-α, and IL-1β, as well as of the anti-inflammatory cytokine TGF-β over the analyzed time period. Indeed a marked and significant reduction of the IL-6 was observed 1 hr after the treatment even though it returned to the basal levels within 24 hr. IL-6 is a regulatory cytokine, having a dual effect. In models of chronic inflammatory diseases, such as collagen-induced arthritis, it has a pro-inflammatory role[26], whereas in models of acute inflammation it exhibits an anti-inflammatory profile.[27] IL-6 also induces the production of most of the acute phase proteins,[28,29] such as C reactive protein (CRP) and Albumin, two important mediators that participate in the amplification of the inflammatory process. Consistently, with the decrease of IL-6, 1 hr after the treatment, no significant increase of CRP and Albumin above the reference values were observed at the same time period. IL-6 plays also a role in leucocyte recruitment *in vivo*.[30] A complex of IL-6 and of the soluble form of its receptor sIL-6Rα can activate endothelial cells to secrete IL-8 and monocyte chemoattractant protein (MCP)-1, and induce the expression of cell adhesion molecules (CAM)[30] which are important biomarkers for inflammatory process. In our study, we tested the serum levels of the soluble forms of cell adhesion molecules (sCAMs) as markers for CAMs, that reflect the expression of membrane-bound adhesion molecules, and the process of inflammation of the vessel wall. The secretion of sVCAM-1 (vascular cell adhesion molecule) and sp-selectin (platelet derived) was not significantly changed after CPC treatment, while that of sE-selectin (endothelial derived) was significantly decreased over all analyzed time period. Many studies have shown that the levels of soluble adhesion molecules increase when the inflammatory response is activated reflecting a generalized endothelial and leukocyte cell activation.[31,32] The observation on the significantly reduction of sp-selectin levels may suggest that the treatment do not activate endothelial cell wall. Endothelial cells play an active role in inflammatory and immune reactions. Similarly to other vascular elements, endothelial cells can produce IL-6 in response to pro-inflammatory signals, while it is not clear whether IL-6 affects the function of normal vascular endothelium.[33] The decrease of sE-selectin circulating levels in comparison to pre values, may be indicative of a reduction of a mild inflammatory state present at the time of the treatment, or related to the partial decrease of IL-6 cytokine which in turn regulates sE-selectin production. On the other hand, the lack of activation of endothelial cells by 5% CPC treatment is also consistent with no significant increase of IL-6 serum levels. Altogether these results indicate that the 5% concentration of CP in the preparation of CPC patches is the best choice, since no additional effects can be obtained with the 10% concentration, and the effects are higher than those observed at 2.5% concentration. A single application with 5% CPC did not induce a significant increase in the serum levels of inflammatory cytokines, acute phase proteins and neuroendocrine stress hormone. These findings suggest that the treatment is safe, having positive effects at the site of CPC application, but no negative side effects at systemic levels (at least related to the analyzed major parameters). Further studies are needed to investigate the immediate and long term effects of CPC therapy after 10 times applications that are commonly used as physiotherapy in clinical practice, in order to test its safety and possible cumulative effects, as well as to understand the intersecting underlying mechanisms activated by Capsaicin and the other identified factors.[34] This step is also needed to better understand the beneficial effects of the CPC application for the treatment of patients suffering from musculoskeletal disorders observed in the clinical practice, in order to be more extensively used in the field of physical medicine therapies.[35-37]

List of acronyms

CP - Cayenne pepper
CPC -Cayenne pepper and kaolin powder cataplasm
CRP - C reactive protein
Human TGF-β1 - Human Tumor Growth Factor-β1
IL-1β - Interleukin 1 beta
IL-6 - Interleukin 6
sVCAM-1 - Vascular Cell Adhesion Molecule-1
sE-selectin - Human sE-Selectin
TNF-α - Tumor Necrosis Factor alpha

Author's contributions

NS, study overview, writing the manuscript, conducting statistical analysis; SL, subject recreation, conducting the measurements, writing the manuscript; JC, subject recreation, conducting the measurements, writing the manuscript; WH, subject recreation, conducting the measurements, writing the manuscript; SZ, subject recreation, conducting the measurements, writing the manuscript.

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Conflict of Interest

The authors declare no conflicts of interests.

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