Hyperalgesia induced by cutaneous freeze injury for testing analgesics in healthy volunteers

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Aims

The early phases of the clinical development of new analgesic agents are severely hindered by a lack of reliable sensitive tests based on experimental pain models. The aim of this study was to assess the ability of a localized hyperalgesia model induced by cutaneous freeze injury to evaluate the pharmacodynamic profile of weak analgesic agents in healthy volunteers.

Method and results

Two groups of 24 healthy volunteers were enrolled in controlled, randomized, double-blind, cross-over studies. After freeze injury, punctate mechanical pain thresholds (MPT) were measured over three consecutive daily sessions to characterize the induced hyperalgesia and compare the effects of (i) oral ibuprofen and acetaminophen and (ii) oral and topical ibuprofen vs. placebo. The freeze injury model provides two types of hyperalgesia, primary and secondary, stable over 72 h. The MPT values (means; 95% confidence interval) in the primary (38.9 g; 34.3, 43.5) and secondary (82.2 g; 81.4, 88.0) areas of hyperalgesia were different from normal skin (107.5 g; 101.5, 115.2). This model clearly showed the antihyperalgesic effect of both systemic and topical ibuprofen (42.1%; 26.6, 61.2 and 33.8%; 16.4, 51.2 of MPT increase, respectively) but not that of acetaminophen.

Conclusion

Cutaneous freeze injury coupled with a von Frey electronic device to assess the mechanical pain threshold is a convenient model that causes no discomfort. The improved sensitivity and stability of this experimental model of hyperalgesia over three consecutive days make it a useful tool for evaluating the efficacy and detecting the potential sites of action of analgesic agents such as nonsteroidal anti-inflammatory drugs in healthy human subjects.

Introduction

It is more difficult to obtain predictive pharmacodynamic data in healthy volunteers in the early phases of clinical development of new analgesics than with some other classes of drugs. There are two main reasons commonly put forward to explain this difficulty. The first is that pain is intrinsically a subjective sensation, presenting a rather high interindividual variability. The second is that

the stimuli used for psychophysical assessment in experimental pain, whether they are thermal, mechanical (pressure, prick or impact), electrical or chemical, activate nociceptors in healthy tissues. Given that most painful clinical situations are accompanied by local hyperalgesia [1, 2], the experimental conditions reported are very different from those encountered in spontaneous pain processes. Several clinical models of central and/

or peripheral sensitization of nociceptive pathways have been developed. Some use topical application [3–5] or intradermal injection [6–9] of capsaicin or mustard oil. Others are based on heat injury [10–14], cold injury [3, 15], ultraviolet (UV) B irradiation [16–18] or a combination of heat plus a topical application of capsaicin [19–21]. Mechanical injury resulting from repetitive pinching of the interdigital web [3, 22, 23] has also been evaluated in this context. However, the use of these models to study the pharmacodynamic profile of analgesics always raises several methodological problems. In most cases, the induced hyperalgesia does not last long enough to cover the duration of action of the analgesic drugs under study. For example, the hyperalgesia induced by a local application of capsaicin or mustard oil is not very stable and lasts only about 1 or 2 h [1, 24]. A longer duration, up to 24 h, can be obtained when hyperalgesia is induced by heat injury plus capsaicin [11, 12]. While this time is generally sufficient to cover the pharmacodynamic response of a single-dose administration, it is usually still too short to perform cross-over clinical trials to compare two or three compounds or doses in the same subject. However, the freeze-induced hyperalgesia described by Kilo *et al.* [15] might meet some of the specific requirements of pharmacological trials. In such situations of repeated testing some authors have raised the interfering effect of habituation which can be alleviated by specific training (25).

The aim of the present study was to determine whether the freeze-induced hyperalgesia model was able to evidence and quantify the pharmacological effect of mild analgesic drugs. Two aspects were considered: first, methodological, to specify the characteristics of the freeze-induced hyperalgesia model; and second, pharmacological, to compare on normal and hyperalgesic skin the effects of acetaminophen (INN, paracetamol) and ibuprofen, which are considered reference drugs for the treatment of mild to moderate pain. These two analgesics were chosen because their mechanisms of action are currently considered as being different. Also, previous studies have shown that it is difficult to demonstrate their analgesic effect by quantitative sensory tests using therapeutic dosages [26, 27]. Systemic and topical routes of administration were used to analyse fully the ability of the model to determine the pharmacological profile of analgesic agents with regard to induced hyperalgesia.

Methods

Study population

The studies were conducted in accordance with the Helsinki Declaration and were approved by the Regional

Ethics Committee (CCPPRB d'Auvergne). Normal healthy patients (ASA class 1) of median age 25 years (range 20–37) participated in the study after giving their written informed consent. A full medical history was recorded and all the volunteers were examined by a physician. None showed any signs of acute or chronic disease. Further exclusion criteria were acute skin disease, excessive alcohol or drug consumption, and contraindications for acetaminophen, ibuprofen or other nonsteroidal anti-inflammatory drugs (NSAIDs).

Induction of cutaneous hyperalgesia

Twenty-two hours before the first day of each experimental session, a small skin area on the anterior glabrous part of the forearm was superficially frozen. The end (1.8 cm²) of a cylindrical copper bar previously cooled to –28 °C was applied to induce a first-degree burn injury. Quantitative sensory testing in this area showed no relevant changes in the 2 h following freezing. A sharply delimited erythema accompanied by a localized hyperalgesia was observed several hours after the skin injury. Interindividual variability in the intensity of the erythema response was low and blisters were never observed. Tissue injury leads to two types of hyperalgesia that differ with respect to their location: a primary hyperalgesia (Z1) limited to the area of injury, and a secondary hyperalgesia (Z2) in undamaged skin surrounding the injury. The level of the induced hyperalgesia was stabilized in all subjects 20 h after freezing. Consequently, we commenced the sensory testing (baseline values of the first session) 22 h after the cold injury. Hyperalgesia was defined as a leftward shift of the stimulus response function, thus involving both allodynia (pain arising from non-noxious stimuli) and hyperalgesia (increased sensitivity to noxious stimuli). We use the term mechanical hyperalgesia as defined in *Textbook of Pain* [28]. In the present study, mechanical hyperalgesia was assessed by punctate-evoked pain. No spontaneous pain in or around the freeze lesion was reported by the healthy volunteers during the study. The contralateral arm was used as a control and was called Z0.

Procedures and evaluations of pain

Skin sensory testing with punctate stimuli was performed by the von Frey technique using the von Frey electronic device (VFE) (Bioseb-France, Id-Tech Bioseb, Chaville, France) (Figure 1) with a constant slope of increasing punctate pressure up to the detection of mechanical pain thresholds (MPT). MPT was defined as the lowest pressure that produced a sensation of pain. Each MPT value was averaged from five separate consecutive measurements at different points in the test

Figure 1

Skin sensory testing to punctate stimulus using the electronic von Frey device, in induced hyperalgesic area

Figure 2

Areas of hyperalgesia induced by freeze. The black arrows indicate the concentric reduction of Z2 surface back to the freezing area, during the 3 days following the injury

areas. All tests were performed by the same investigator. The measurement of MPT was carried out within a control skin zone (Z0) on the contralateral forearm of the injured arm and within two concentric hyperalgesic zones: the central primary hyperalgesic zone (Z1) (injured freeze skin zone) and the secondary hyperalgesic zone (Z2) surrounding Z1. The secondary hyperalgesic area was determined with the classical Semmes–Weinstein monofilament (using the 4.08 monofilament) by stimulation applied radially around the site of the freeze injury (Figure 2). Stimulation was started well outside the hyperalgesic area, where no sensation of pain was experienced, and continued centripetally in 5-mm steps towards the site of freeze injury until the subject reported a definite change in sensation. The border was marked on the skin and the mapping was traced onto a clear acetate sheet for later measurements. The surfaces of both areas were quantified with a vector algorithm using a SONY digital still camera (MVC-FD7; Sony Corporation, Japan) and PC-based morphometry system software (Image Tools, UTHSCSA, USA).

Design of the study

Two experiments, each involving a different group of 24 healthy male volunteers, were carried out according to a randomized (using a balanced Latin square design), double-blind, placebo-controlled, cross-over study design. As this was an exploratory study on a new model, the number of subjects was estimated based on previous data taking a first type risk (α) of 5% and a

power ($1-\beta$) of 80% to discriminate between two formulations having a minimum difference of 20%.

All the subjects participated in three sessions on three consecutive days (22 h, 46 h and 70 h post injury). They had been familiarized beforehand with the methods of quantitative sensory testing and rating procedures through six preliminary training sessions.

- 1 The first study enabled us to (i) determine the characteristics of the hyperalgesia induced by the freeze injury and (ii) comparatively evaluate the efficacy of analgesic drugs on normal (Z0) and freeze-injured skin (Z1). Volunteers were given a single oral dose of ibuprofen (400 mg, Gelufene®; French Pharmaceutical Corporation, Melun, France) or acetaminophen (1000 mg, Geluprane®; Theraplix, Sanofi-Aventis Group, Paris, France) or a placebo, on three separate days and at 24-h intervals.
- 2 The second study was performed to determine the ability of the model to identify the preferential site of action of analgesics. For this purpose, the antihyperalgesic effects of ibuprofen were evaluated on primary (Z1) and secondary (Z2) hyperalgesic zones after local administration (200 mg topical gel formulation of 5% ibuprofen: Ibutop®; Chefaro-Ardeval, Montrouge, France) compared with the placebo (200 mg topical gel) and after oral ibuprofen (400 mg Gelufene®; French Pharmaceutical Corporation) used as a positive reference. Topical gel formulations (2 mg cm^{-2}) were applied by gentle massage for 1 min on the skin surface broadly covering the hyperalgesic areas.

Each study was performed in a quiet room. All the sessions took place at the same time of day to minimize possible circadian influences on pain ratings. MPTs were determined twice before each drug administration (baseline values), and then 60 min, 120 min and 180 min after medication.

Expression of results and statistical analysis

Statistical analysis was performed using SAS Software (SAS 8.1; SAS Institute, Cary, NC, USA). The effects of the analgesic treatments were expressed either as the raw data for the analysis before treatments (baseline values of MPT) or as the percentage of variation of MPT individual baseline value to assess treatment effect. The global effect (0–180 min) of the analgesic drugs was assessed by calculating the mean areas under the time–course curves (AUC) of percentage changes in MPT of each volunteer, using the trapezoidal method.

Data are presented as means together with their 95% confidence intervals (CI). For reasons of clarity, the results presented in the figures are expressed as means $\pm$ SEM.

Normal distribution of data was checked using the Kolmogorov–Smirnov test. Changes in MPT were assessed by a separate ANOVA. Statistical analysis took into account treatment (ibuprofen, acetaminophen and placebo) and period (session 1, 2 and 3) for each site (Z1, Z2 and Z0 areas). After ANOVA analysis, *post hoc* test comparisons were made using the Tukey test or Student's *t*-test between the combinations of parameters. The significance limit was $P < 0.05$.

Results

Characteristics of induced hyperalgesia

Areas of hyperalgesia The areas of the zones of primary and secondary induced hyperalgesia were determined over three consecutive sessions, S1 (22 h post injury), S2 (46 h post injury) and S3 (70 h post injury). The surface areas were, respectively: 2.0 (1.8, 2.2), 1.8 (1.2, 2.4) and 1.8 (1.6, 2.2) cm² for Z1 and 17.7 (10.9, 24.4), 16.1 (7.7, 24.5) and 12.4 (5.0, 19.8) cm² for Z2. We noted that the Z2 area shrank concentrically back to the freezing site, but a ring at least 1 cm wide in which the MPT could be measured remained available in all the subjects for 72 h following injury.

Assessment of the stability of hyperalgesia The MPT values over the three experimental days (sessions 1, 2 and 3) were measured using the punctate mechanical stimuli on each tested skin area (Z0, Z1 and Z2). From the coefficient of variation (Table 1) we noted that MPT value dispersion for each session remained moderate.

Table 1

Coefficient of variation of mechanical pain thresholds (MPT) evaluated by using the von Frey electronic (VFE) method on areas of hyperalgesia (primary Z1, and secondary Z2) and control skin (Z0) site over the three experimental sessions

Area	MPT measurement method	Coefficient of variation		
		Session 1	Session 2	Session 3
Z1	VFE	63	54	54
Z2	VFE	49	35	35
Z0	VFE	28	29	29

Figure 3

Mechanical pain thresholds (MPT) (g) determined using the von Frey electronic device (VFE) method over the three consecutive experimental sessions on control skin (Z0) and on hyperalgesic zones (primary Z1, and secondary Z2). Data are expressed as means $\pm$ SEM. With respect to each area, the MPT values over the three sessions were not significantly different. The mean control MPT on Z0, Z1 and Z2 areas were significantly different ($0.05 < P < 0.001$) from each other for each session (* vs. Z0 and # vs. Z1). Session 1 (□), Session 2 (▨), Session 3 (■)

The time course of MPT baseline values for the three experimental sessions evaluated in the Z0, Z1 and Z2 zones are shown in Figure 3. Freeze injury-induced hyperalgesia was characterized by a significant decrease in the pain threshold in the Z1 and Z2 zones vs. Z0. The mean MPT values over the three sessions of the first study in Z0 (107.5 g; 101.5, 115.2), Z1 (38.9 g; 34.3, 43.5) and Z2 (82.2 g; 81.4, 88.0) were significantly different ($P < 0.0001$). For each area, ANOVA showed neither period nor sequence effect, thus there were no significant variations in the MPT values over the three experimental sessions, thereby indicating an absence of

carry-over effect and a sufficiently long wash-out period. This is confirmed by the published apparent terminal half-lives for both investigated compounds that are short (2 h and 4 h for ibuprofen and acetaminophen, respectively) [29]. The wash-out period of 24 h was sufficient to eliminate more than 99% of the compound. Furthermore, during each session the MPT values recorded after placebo showed no significant shift (Figures 4 and 5) over the 180 min following administration.

Effects of analgesic drugs

Effects of systemic administrations The effects of oral administration of ibuprofen, acetaminophen and placebo evaluated comparatively in normal (Z0) and in freeze-injured skin (Z1) are illustrated in Figure 4. Before each treatment, the MPT values were not significantly different in Z0 control skin (109 g; 95.6, 122.6, 109.1 g; 96.4, 121.7 and 106.9 g; 95.6, 118.2 before ibuprofen, acetaminophen and placebo, respectively) or in Z1 injured skin (36.4 g; 24.9, 48.6, 43.4 g; 32.7, 54.1 and 39.6 g; 30.8, 48.5, respectively). The effects of analgesics, analysed as the percentage change from MPT baseline values, are reported in Figure 4A. Neither placebo nor acetaminophen had any significant effect on

either of the two tested areas, and ibuprofen had no significant effect on normal skin. In contrast, ibuprofen significantly increased ($P < 0.05$ vs. placebo) the MPT in the injured skin area (Z1) over 180 min after drug intake (maximum effect 40.9%; 20.1, 59.6). The overall effects of ibuprofen, acetaminophen and placebo were assessed by the areas under the time-course curves ($AUC_{0-180 \text{ min}}$) for percentage change in MPT (Figure 4B). No change in AUC was observed in the normal skin area after analgesic treatment. In contrast, there was a highly significant treatment effect in the hyperalgesic area (Z1) ($P < 0.004$), with differences between ibuprofen and acetaminophen ($P = 0.015$) and ibuprofen and placebo ($P = 0.001$). Acetaminophen was not statistically different from placebo ($P = 0.366$). Moreover, the overall efficacy of oral ibuprofen was 1.9-fold higher than that of acetaminophen and 3.2-fold higher than that of placebo.

Effects of topical applications In comparison with placebo, oral and topical administration of ibuprofen (Figure 5A) significantly increased MPT in area Z1. The maximum increase was 42.1% (26.6, 61.2) and 33.8% (16.4, 51.2) for oral and topical application vs. placebo, respectively. However, no statistical difference was observed between the effects of these two forms. In area

Figure 4

Analgesic effects of oral ibuprofen (per os) (▨) (400 mg), acetaminophen (per os) (■) (1000 mg) and placebo (□) on uninjured skin site (Z0) and on freeze-injured skin (primary hyperalgesia area, Z1). (A) Mechanical pain thresholds (MPT) (g) determined using the von Frey electronic device (VFE) method are expressed as percentage changes from basal value. The antihyperalgesic effect of ibuprofen was significantly different from placebo for the Z1 area ($*P < 0.01$). For the other treatment area, no significant difference was seen. (B) Global analgesic effect of treatments reported in histogram form of area under time–pain curve (AUC). For primary hyperalgesia Z1 ibuprofen was different from placebo ($*P < 0.01$) and from acetaminophen ($\#P < 0.01$). Data are expressed as means $\pm$ SEM

Figure 5

Analgesic effects of ibuprofen in topical application (200 mg of gel 5%) and oral administration (400 mg) and placebo on primary (Z1) and secondary (Z2) hyperalgesia areas. Ibuprofen (per os) (▨), ibuprofen (topical gel) (■), placebo (□). (A) Mechanical pain thresholds (MPT) determined using the von Frey electronic device (VFE) method were expressed as percentage changes from baseline values. The effects of oral and topical ibuprofen were significantly different from placebo for area Z1 (* $P < 0.001$ and $P < 0.01$, respectively). For the Z2 area, only the effects of oral ibuprofen were significantly different (* $P < 0.005$) from placebo. (B) Global analgesic effect of treatments reported in histogram form of area under time-pain curve (AUC) confirms the effects of oral and topical ibuprofen for areas Z1 and Z2. Data are expressed as means $\pm$ SEM

Z2, a significant increase in MPT values was also observed with oral ibuprofen (maximum increase 22.7%; 12.0, 33.5). Unlike Z1, the effect of the topical gel (maximum increase 10.8%; 1.21, 22.9) was not significantly different from placebo. The overall effects of ibuprofen, as reflected by AUC values (Figure 5B), confirmed the antihyperalgesic efficacy of both forms in both areas. In the primary hyperalgesic area, a treatment effect was observed between both ibuprofen treatments and placebo ($P = 0.001$ and $P = 0.017$ for oral and topical application vs. placebo, respectively), with no statistical difference between ibuprofen treatments ($P = 0.3$). For the secondary hyperalgesic area, the AUC increased significantly only with oral ibuprofen ($P = 0.005$) and this score was statistically different from that of ibuprofen topical gel ($P = 0.04$). The overall effects of both forms of ibuprofen were significantly higher in the primary hyperalgesic area than in the secondary hyperalgesic area.

Discussion

This study was conducted with a view to improving the sensitivity, reproducibility and predictability of experimental pain tests. It was designed to investigate the pharmacodynamic profile of analgesic compounds in healthy volunteers. This model of freeze injury-induced hyperalgesia, which had not been previously tested with this aim, clearly evidenced the stability of the induced

hyperalgesia over 72 h. In contrast to the short steady-state of most human experimental pain models [30], which are thus ill-suited to assessing the pharmacodynamic profile of long-acting analgesics or to comparing several analgesics or doses in studies with subjects used as their own controls [27], the time-course of the present injury model allows a cross-over design scheduled on three consecutive days. As shown by repeated measurements of baseline values over 72 h after cold injury, the induced hyperalgesia remains quite stable in both Z1 and Z2 areas. Furthermore, MPT recording over 180 min following placebo intake shows that there is no shift due to habituation effect if one takes care to provide good training before inclusion in the study. The induction of the freeze skin injury is brief (8 s) and almost painless, unlike heat injury, which is fairly uncomfortable (7 min at 47 °C [13]). As previously reported by Kilo *et al.* [15], no spontaneous pain, either immediately after the freeze injury or during the following days, is experienced by the subjects. This feature is very important not only ethically but also methodologically, because it avoids any pain phenomena that might interfere with the evaluation of pain thresholds [31–33]. However, a slight residual brown pigmentation was observed in the injured area, lasting a few weeks or even 1–2 months in a few subjects, especially those of dark complexion. This tanning effect, which is also observed in other models of experimental hyperalgesia induced

by heat or UVA injuries, can be largely avoided by recommending sun protection over the 2 weeks following the freeze injury.

Many experimental studies in healthy volunteers have failed to determine the pharmacodynamic profile of agents with moderate analgesic activity [26, 27] in clinically relevant doses. In contrast, our data show that the cutaneous freeze model coupled with the electronic von Frey to assess MPT is able to demonstrate the effect of NSAIDs given at analgesic dosage. The von Frey electronic method shows some advantages compared with the conventional monofilament method. It allows a continuous measurement to be obtained, is easy to use and gives a measurement in a single operation. In addition, the use of a disposable measurement device prevents any possible interindividual contamination.

Consistent with the results obtained with higher doses of ibuprofen [26, 34], the present study with 400 mg oral ibuprofen showed no relevant effect on normal skin (Z0), but a significant increase in the mechanical pain threshold was observed in primary hyperalgesia over 180 min following drug intake. These results agree with those published by Petersen *et al.* [27] indicating that the hyperalgesia is essential in the experimental demonstration of the analgesic effect of NSAIDs. Unlike cold pressor-induced pain, which is sensitive to the effect of acetaminophen [30] but not to that of ibuprofen [35], our model appears to be less suitable for evaluating the analgesic effect of acetaminophen with standard therapeutic dosages than of ibuprofen. The difference observed between the effects of these two drugs may reflect differences in their mechanisms of action, although that of acetaminophen is still unclear [36–40].

Another strong advantage of this model is its ability to distinguish between two types of hyperalgesia: a primary hyperalgesia (Z1) at the site of the freeze injury, clearly delimited by skin reddening, and a surrounding secondary hyperalgesia (Z2) of apparently undamaged tissue. Action on these two types of hyperalgesia, which may be due to different mechanisms [14], could provide insight into the peripheral and central components of the effects of analgesic drugs. As stated in our results, the surface of Z2 shrank concentrically during the 3 days following the injury. Therefore, the measurements have to be carried out inside a ring at least 10 mm wide in order to obtain a reliable assessment of pain thresholds. Given that hyperalgesia lasts for 3 days, the model proposed allows comparison of various administrations or drugs. However, the pharmacokinetic characteristics of the products must be taken into account, as we did, in order to avoid any carry-over effect.

Unlike heat-induced hyperalgesia, which is not

altered by either topical or systemic ibuprofen, even at higher dosages than those used in this study [26], we observed a reduction in pain after systemic and topical ibuprofen in both hyperalgesic areas. An antihyperalgesic response has been also observed at the site of inflammation with high doses of oral ibuprofen [3] and oral and topical diclofenac [41]. In the present study, the slight difference in the magnitude of the analgesic effects of systemic and topical ibuprofen may be related to the pharmacokinetic properties of ibuprofen, depending on the administration route. While systemically administered ibuprofen is able to reach an effective concentration in the central nervous system after crossing the blood–brain barrier, a central effect cannot reasonably be expected with a topical application of ibuprofen, for which bioavailability is around 0.6%, compared with the oral route [42]. On the other hand, in the same pharmacokinetic study using a microdialysis technique, Tegeder *et al.* [42] showed that topical administration of ibuprofen leads to greater concentrations of active compound in the underlying subcutaneous tissues than after oral administration. Consequently, the effect of topical ibuprofen on the primary hyperalgesia area, which appears rapidly, can be mainly explained by a peripheral site of action. This effect may mainly involve the blockade of the local release of algogenic substances, such as prostaglandins, in the injured area. In a heat injury model [26], the efficacy of topical application of ibuprofen was not observed after a single application, and became apparent only after repeated applications [12, 43]. This lag was interpreted by the authors as being the time required for accumulation of NSAID at the site of injury. The subcutaneous concentration required to obtain an anti-inflammatory effect may be much higher than the concentration required to induce an analgesic effect. On this hypothesis, the pain relief observed promptly after topical application in our study was due to a local antinociceptive effect rather than an anti-inflammatory effect. The fairly equivalent efficacy we showed for topical and oral ibuprofen is in line with a recently published meta-analysis [44] and review [45] about the use of NSAIDs on musculoskeletal pain and rheumatic diseases. In addition, our results are in accordance with those obtained with diclofenac [41], which lead the authors to suggest that a central mechanism may be involved in the antihyperalgesic effect of orally administered NSAIDs.

To conclude, freeze injury used as a pain model showed high between-day repeatability in pain responses to mechanical stimuli over three consecutive days in both primary and secondary hyperalgesia areas. This model is thus well suited to investigating the phar-

macological profile of systemic or topical analgesics in clinically relevant doses, and especially for assessing long-acting effects and/or performing cross-over studies. Even if it is not possible to provide evidence of a total overlap with pathological situations, this model may also be useful for clarifying the pathophysiological mechanisms of hyperalgesia underlying the different types of symptoms observed in patients with acute or chronic pain.

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