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TECHNICAL PAPER

QUANTITATIVE CHARACTERIZATION OF A REPEATED ACUTE JOINT INFLAMMATION MODEL IN RATS

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SUMMARY

1. Chronic pain owing to arthritis is a major clinical problem worldwide. To study the underlying pathological mechanisms of chronic pain and the effectiveness of different treatments, a number of experimental models have been developed over the years.

2. We introduced a new subchronic inflammatory model by repeated unilateral administration of carrageenan into the ankle joint of rats, and investigated the degree and the time-course of the oedema, and thermal and mechanical hyperalgesia.

3. Carrageenan (450 μg) was injected on three occasions (on days 1, 4 and 7), and the resulting oedema, thermal hyperalgesia (paw withdrawal test) and weight load were characterized in voluntarily walking rats daily for 15 days. The effect of diclofenac sodium (3 mg/kg orally daily for 15 days) was also determined.

4. Repetitive administration of carrageenan caused fluctuating oedema and pain responses, which did not normalize within 3 days. Exacerbated inflammatory oedema was observed after the second and third injections. Oedema and a decreased weight load of the inflamed paw were observed throughout the investigation period, and paw withdrawal thresholds to noxious thermal stimuli returned to baseline pre-carrageenan values from Day 13.

5. Oral diclofenac (3 mg/kg daily for 15 days) significantly decreased oedema within a few days (Day 3), whereas its anti-allodynic effect developed only several days later (Day 9). However, diclofenac at the applied dose did not influence the thermal hyperalgesia.

6. The results suggest that the repeated administration of carrageenan might be a suitable model for determining the effects of long-lasting treatment.

Key words: arthritis, chronic pain model, diclofenac, gait analysis, hyperalgesia, oedema.

INTRODUCTION

Chronic pain owing to arthritis is a major clinical problem worldwide. To study the underlying pathological mechanisms of chronic pain and the effectiveness of different treatments, a number of experimental models have been developed over the years. One of the most common animal models of arthritis is the inflammation induced by complete Freud's adjuvant (CFA) (heat-killed *Mycobacterium butyricum*). Systemic injection of CFA induces long-lasting inflammation in multiple joints, resulting in severe conditions in which polyarthritis is accompanied by widespread systemic disease, complicating the interpretation of the data.[1] To our knowledge, very few models of mono-arthritis have been described yet. The most widely used method for mono-arthritis induction is the local administration of CFA in low doses. However, an increase in the circumference of the joint develops after several days (around Day 3), and it cannot be determined whether systemic inflammation occurs in some animals.[2] The injection of CFA into the tibiotarsal joint gives rise to intensive and persistent mechanical and thermal hyperalgesia with duration of over 4 weeks, supposing that it causes long-lasting suffering.[3] Therefore, despite the advantages of this model, the severe pain associated with the disease provokes ethical concern, which has prompted several workers to develop other models of mono-arthritis. Furthermore, it is well known that the course of rheumatoid arthritis is intermittent, therefore a model with a consistent pattern would be more advantageous. Carrageenan is a frequently used agent for the induction of experimental inflammation and inflammatory pain, which is considered relevant to clinically important inflammatory pain states. In some studies, researchers have applied two carrageenan injections into the plantar surface 7 days apart,[4,5] and investigated the pain and inflammation of the paw, finding that the rats recovered completely. Only one article has reported on injections of carrageenan repeated twice within a shorter interval (72 h) into the knee joint (300 μg) on the mechanical nociceptive sensitivity in rats.[6] These studies suggest that repeated application of carrageenan might be a valuable model for chronic/subchronic inflammation.

The main purpose of the present study was to characterize the extent of subchronic single-joint inflammation induced by repeatedly injected carrageenan. The second goal was to investigate whether a recently introduced device (developed to characterize the weight load in voluntarily walking rats) is a reliable method for the determination of pain sensation after ankle joint inflammation. Finally, we also tested the anti-inflammatory and antinociceptive effects of diclofenac, which is a well-known non-steroidal anti-inflammatory drug (NSAID).

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Table 1 Treatment schedule. Bodyweight and baseline values of the ankle cross-section area on Day 1 before and 3 h after carrageenan injection

Group	*n*	Nociceptive test	Oedema test	Weight (g)				Ankle cross-section area (mm^2)			
								Pre-carragenan		Post-carrageenan	
				Day 1	Day 1	Day 15	Day 15	Right	Left	Right	Left
Control	7	PWD	+	254 ± 7.5	257 ± 5.2	312 ± 7.7	314 ± 5.7	47 ± 2.5	47 ± 2.0	95 ± 3.1*	46 ± 2.3
Control	6	Weight load (g)	+	262 ± 7.3		316 ± 9.1		52 ± 0.7	52 ± 1.3	97 ± 3.1*	51 ± 1.7
Diclofenac	9	PWD	+	267 ± 13.6	262 ± 8.5	341 ± 9.0	330 ± 6.9	50 ± 2.2	51 ± 2.2	94 ± 5.3*	51 ± 1.4
Diclofenac	6	Weight load (g)	+	254 ± 6.6		312 ± 6.2		53 ± 1.4	50 ± 1.5	94 ± 1.2*	49 ± 0.5

*Significantly different compared with the pre-carrageenan value ($P < 0.05$).
PWD, paw withdrawal; *n*, number of animals.

METHODS

After institutional ethical approval had been obtained (Institutional Animal Care Committee of the Faculty of Medicine, University of Szeged), male Wistar rats (Charles River strain; Bioplan, Budapest, Hungary) were housed in groups of five or six animals per cage, with free access to food and water, and keeping to the natural light : dark cycle.

The following drugs were administered: γ-carrageenan (Sigma-Aldrich Kft., Budapest, Hungary) dissolved in physiological saline and diclofenac sodium (Voltaren 75 mg/3 mL injection; Novartis Hungária Kft., Budapest, Hungary). Diclofenac sodium was administered orally daily for 15 days through a gastric tube (3 mg/kg per day; 2 mL/kg volume, diluted with saline). This dose has significant anti-inflammatory potency in rats.[7,8] The control group received the same volume of physiological saline. The first injection was applied 3 h after the carrageenan injection.

To induce arthritis, γ-carrageenan (450 μg/30 μL) was injected into the tibiotarsal joint cavity of the right hind leg on days 1, 4 and 7. All treatments were given to gently restrained conscious animals, using a 27-gauge needle, and without anaesthesia so as to exclude any drug interaction. These injections did not elicit any signs of major distress, such as audible vocalizations or attempts to bite the experimenter.

The rats were randomly assigned to the control and treatment groups, and all of the entered rats completed the study (Table 1).

To follow the changes in the size of the inflamed joint, we measured the antero-posterior and medio-lateral aspects of the ankle joint with an electronic calliper. The cross-section area was calculated by the formula $a \times b \times \pi$, where *a* and *b* are the radius in the two aspects.

Two types of nociceptive tests were performed. In the first series of experiments, thermal hyperalgesia was assessed by means of the paw withdrawal (PWD) test; and in the second, weight load test was used to measure mechanical hyperalgesia. The two tests were performed in different animals to decrease the level of stress and pain stimuli, which might have influenced the results.

In the PWD test, the rats were placed on a glass surface in a plastic chamber and allowed to acclimatize to their environment for 20 min before the hind paw withdrawal latencies were determined. Heat stimulus was directed onto the plantar surface of each hind paw. Cut-off time was set at 20 s to avoid tissue damage. A detailed description of this method has been published elsewhere.[9] The weight load test on each leg was performed according to a model introduced recently by Min *et al.*[10] The apparatus consists of a starting box, a path and an arrival box. The path of the apparatus is constructed in such a way that while a rat is walking through it, the weight load on a given leg of the animal can be monitored at a maximum of four different spots along the path. The output of each load cell is fed to a digital amplifier for appropriate amplification and filtering. The processed signal is sent to a personal computer and plotted as a time-course curve. The rats were trained in the apparatus for three days before the first carrageenan injection was administered. The test was repeated three times consecutively each day. Both pieces of equipment were homemade as the cited authors suggested.

The PWD latencies or the weight load and the diameters were obtained consecutively on days 1, 4 and 7 in the morning (AM) before and again 3 h after (PM) the carrageenan injections. On the other days, the measurements were made once daily in the afternoon for 15 days. Throughout the study, the measurements were carried out in a double-blind fashion.

Data are presented as the mean±SEM. As the weight applied to the one leg depends on the bodyweight, the weight load value was expressed as a percentage of bodyweight (relative weight load; RWL).

Analysis of variance (ANOVA) of the data for repeated measures was used to examine for any overall effects, followed by the Fisher LSD test. A probability level of 0.05 was considered significant.

RESULTS

There were no significant differences in weight between the groups on days 1 and 15, and the weights of the animals increased similarly and significantly in each group during the 2 weeks (Table 1), suggesting that diclofenac did not cause severe gastric toxicity. Similarly, the basal values of the different parameters (i.e. weight load, PWD latency and ankle cross-section area) did not differ significantly between the control and the diclofenac-treated groups (Tables 1,2); accordingly, we did not normalize the data to the weight of the animals, except for the weight load (RWL).

Oedema

The first injection of carrageenan caused a similar degree of oedema in all groups on the inflamed side 3 h after the injection, but it did not influence the cross-section area on the contralateral side (Table 1). As there were no significant differences between the two control and two diclofenac-treated groups at any time throughout the period, we merged these data for further analysis.

With regard to the changes in time in the control group, we found that the oedema was maximal 3 h and 24 h after the first carrageenan injection; it decreased significantly on days 3 and 4, but did not normalize. The second carrageenan injection caused a higher degree of oedema on days 4, 5 and 6; that is, the cross-section was significantly larger when compared with that on days 1, 2 and 3, respectively (Fig. 1). The third carrageenan injection caused a similar degree of oedema as that by the second application. The oedema decreased continuously from Day 9, but significant differences could be observed between the two sides throughout the period. No significant changes were observed on the normal side.

Considering the diclofenac-treated group, the second and third injections of carrageenan did not cause an increased level of oedema, but significant differences were observed between the two sides during the whole period. The comparison revealed that there were

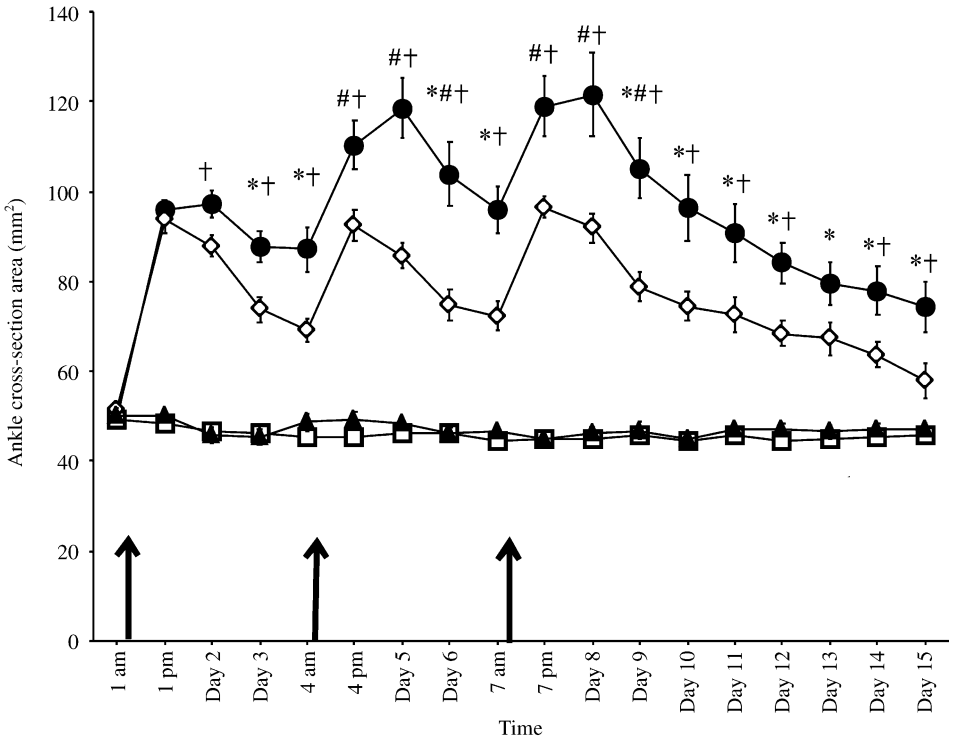

Fig. 1 Ankle cross-section area (mm^2) of the inflamed (I) and non-inflamed (NI) hind paws of the control and diclofenac-treated (3 mg/kg per day orally for 15 days) animals. (●), Control I; (□), control NI; (◇), diclofenac I; and (▲), diclofenac NI. *Significantly higher cross-section area compared with the first carrageenan injection value ($P < 0.05$). †Significant differences between control and diclofenac-treated animals ($P < 0.05$). #Significantly different compared with carrageenan values measured 3 h after the injection ($P < 0.05$). The arrows show the time points at which carrageenan was injected.

Table 2 Baseline paw withdrawal (PWD) latencies and weight load on Day 1 before and 3 h after carrageenan injection

Group	*n*	Parameters	Pre-carrageenan				Post-carrageenan			
			Right FP	Left FP	Right HP	Left HP	Right FP	Left FP	Right HP	Left HP
Control	7	PWD latency (s)			9.4 ± 0.59	10.3 ± 0.65			3.6 ± 0.60†	11.1 ± 0.77
Control	6	Weight load (g)	109.6 ± 4.18*	111.5 ± 3.19*	137.8 ± 3.70	135.2 ± 2.35	101.0 ± 6.42	128.5 ± 5.07†	100.4 ± 8.23†	160.2 ± 3.85†
Diclofenac	9	PWD latency (s)			9.8 ± 0.76	9.3 ± 0.50			4.1 ± 0.63†	10.8 ± 0.69
Diclofenac	6	Weight load (g)	114.9 ± 3.13*	104.0 ± 2.20*	138.6 ± 4.82	140.4 ± 4.47	102.3 ± 7.31	122.1 ± 4.02†	86.6 ± 7.35†	162.1 ± 3.32†

*Significantly different from the hind paw ($P < 0.05$).
†Significantly different from the pre-carrageenan value ($P < 0.05$).
FP, fore paw; HP, hind paw; *n*, number of animals.

significant differences between the two groups from Day 3; that is, the ankle cross-section area was significantly larger in the control group than in the diclofenac-treated group. There were no significant differences between the groups on the normal side.

Paw withdrawal test

All three γ-carrageenan injections significantly decreased the PWD latency on the inflamed side in both groups, whereas the thermal sensitivity of the unaffected paw did not alter significantly throughout the experiment (Table 2; Fig. 2). There were no significant differences in the hyperalgesic effects between the first, second and third injections of carrageenan. Paw withdrawal latency increased from days 2 to 4 and days 5 to 6 and continuously from Day 8. There were no significant differences between the two sides on the mornings of days 6 and 7 and from Day 13 until the end. In terms of diclofenac treatment, the changes were similar to those in the control group; that is, there were no significant differences between the two groups at any time points (Fig. 2).

Weight load test

Baseline weight load values were similar for both hind paws, but the load borne by the fore paws was significantly less than that borne by the hind paws (Table 2). Carrageenan injections resulted in a significant reduction of RWL for the injected leg after 3 h, and the decreased RWL persisted throughout the investigation period in the control group, although its degree fluctuated (Fig. 3). The most intensive RWL decrease was observed 1 day after the injections; there was then a continuous improvement in the days that followed. The repetition of carrageenan administration did not produce more aggravated motor impairment.

Regarding the control left (non-inflamed) hind paw, an increase in the RWL was observed from days 1 to 5. The RWL of the right fore paw decreased significantly from Day 2 and persisted until Day 12, and the load of the left fore paw was significantly increased only 3 h after the first carrageenan injection.

The effect of diclofenac on the RWL on the inflamed side was expressed slowly; that is, significant differences between the two

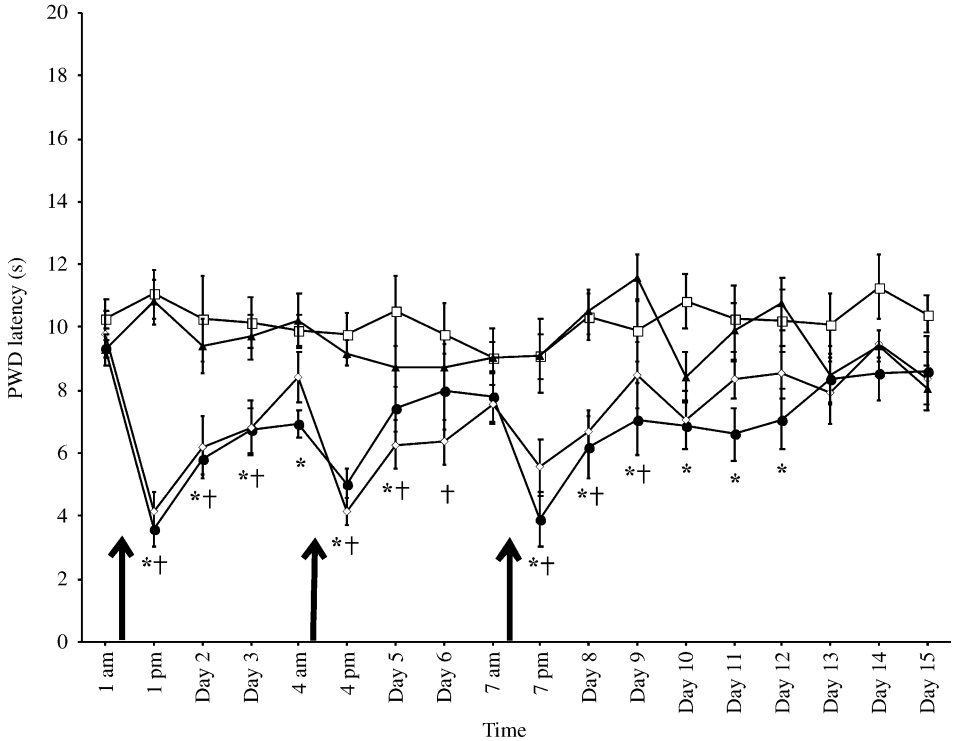

Fig. 2 The time-response curves indicate the paw withdrawal (PWD) latencies on the inflamed (I) and non-inflamed (NI) hind paws of the control and diclofenac-treated animals. (●), Control I; (□), control NI; (◇), diclofenac I; and (▲), diclofenac NI. The symbols denote a significant ($P < 0.05$) difference between the inflamed and non-inflamed sides of the control (*) and diclofenac-treated (†) groups. The arrows show the time points at which carrageenan was injected.

groups were observed only from Day 9, and the mechanical hyperalgesia was relieved from Day 13, suggesting a faster recovery. For the other paws, there were no significant differences between the two groups. By contrast, in the diclofenac-treated groups we did not observe the reciprocal changes in the RWL on the contralateral side.

DISCUSSION

The present data show that the repeated administration of carrageenan into the right ankle joint (450 μg on days 1, 4 and 7) caused fluctuating oedema, and thermal and mechanical hyperalgesia. The degree of oedema was exacerbated after the second and third injections, but the thermal and mechanical hyperalgesia did not change. The signs of inflammation gradually decreased after the last carrageenan injection, thus the reduction in oedema and thermal hyperalgesia began on the second day after the injections, but the gait impairment was more persistent. Orally administered diclofenac sodium resulted in a significant decrease in oedema within a few (i.e. three) days, whereas its anti-allodynic effect developed only several days later (Day 9). However, diclofenac treatment at the applied dosage did not influence thermal hyperalgesia.

More than 10 years ago, Guilbaud *et al.* demonstrated that the first inflammation enhances the pain behaviour (but not the oedema) relative to a second inflammation induced in either the ipsilateral or the contralateral hind paw.[4,5] We found the opposite result in our model; that is, we observed exacerbated oedema, but not increased thermal and mechanical hyperalgesia. The reason for the inconsistent results could be because of the difference in the interval between injections after total recovery (which was 3 days apart in the present study) compared with 7 days apart.[5] Another explanation might be the difference in the route of carrageenan injection (intraplantar *vs* intra-articular), as it is well known that the mechanisms of pain induced in the various tissue types (e.g. muscle, joint and skin) are different.[11,12] Thus, the central projections of the primary afferents innervating muscles and joints are predominantly directed to lamina I and deeper laminas of the dorsal horn, whereas those of the cutaneous tissues also project to lamina II.[13]

Single administration of carrageenan into a joint is a well-known inflammatory model in rats.[11,14,15] A recent study investigated the long-term effects of 300, 1000 or 3000 μg carrageenan injected into the knee joint of rats,[12] and the hyperalgesia in response to mechanical and thermal stimuli was assessed at various time points for 8 weeks. The highest dose of carrageenan produced hyperalgesia ipsilaterally and lasted for 7–8 weeks; however, it spread to the contralateral side 1–2 weeks after the injection. A dose of 1000 μg carrageenan produced a shorter-lasting hyperalgesia that remained ipsilateral. A dose of 300 μg carrageenan caused only a short-lasting (24 h) hyperalgesia. Thus, the degree and the duration of oedema and pain was dependent upon the dose applied. As the carrageenan was injected into the knee joint and the pain stimuli were applied to the plantar surface of the hind paws, we determined secondary thermal and mechanical hyperalgesia. The degree of hyperalgesia seemed to be high, suggesting that higher doses of carrageenan induced very severe and long-lasting pain in the animals. In the present study, we also determined the level of secondary thermal hyperalgesia (stimulus onto the plantar surface of the hind paw *vs* pain induction in the joint) and found that decreases in the paw withdrawal latencies of only about 2–3 s were observed 2 days after the carrageenan injections, suggesting a lower level of suffering. This is also supported by the observation that there was normal weight gain (Table 1) and the animals walked voluntarily.

In terms of the inflammatory potential of CFA, Stein *et al.* investigated its nociceptive effect after unilateral intraplantar injection.[16] The main difference when compared with our model was that the inflammation was apparent within 12 h; it increased progressively, reached a peak at Day 16 and then slowly subsided. The inflammation

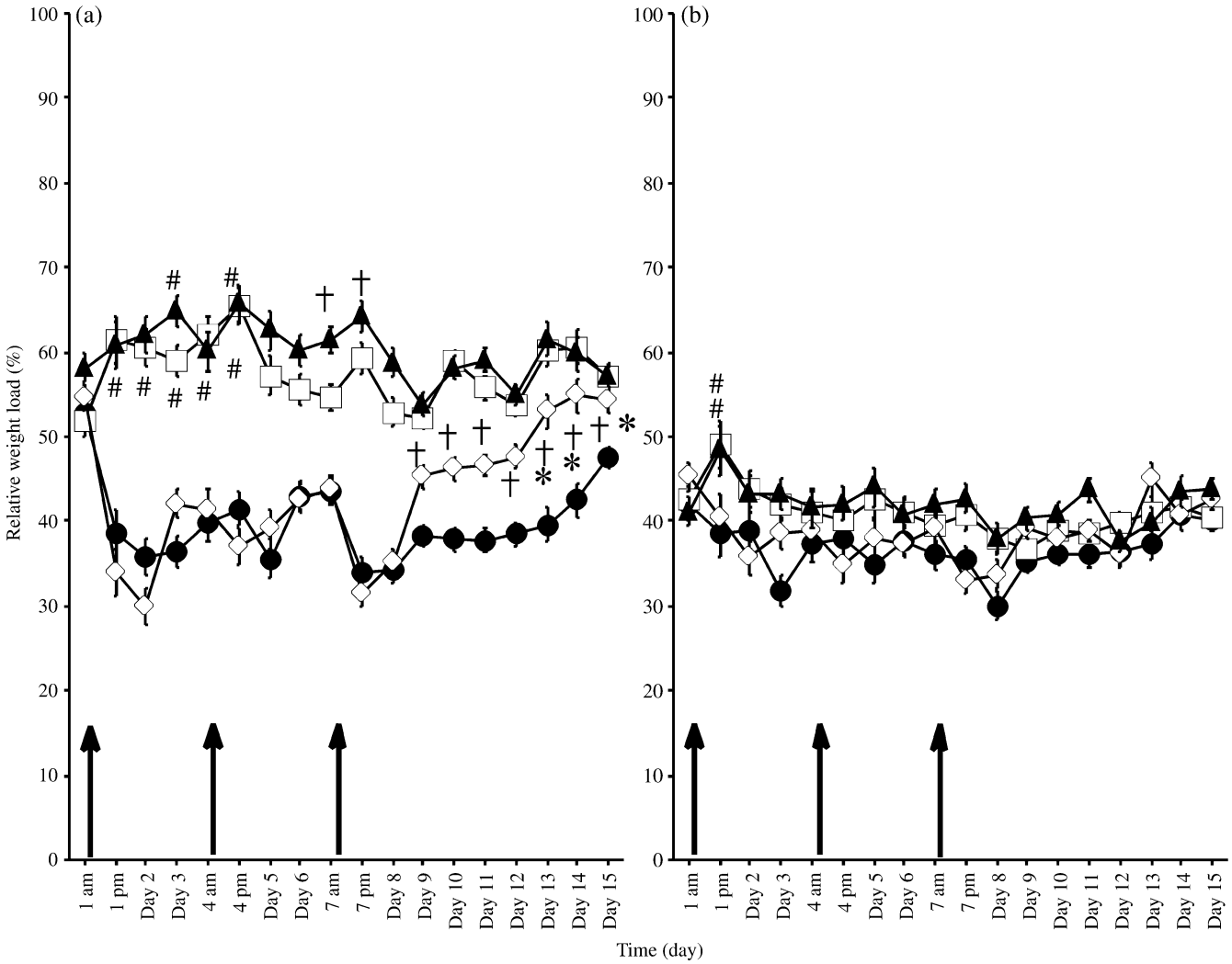

Fig. 3 The curves indicate the relative weight load of the (a) hind paws and (b) fore paws of the control and diclofenac-treated animals. (●), Control right; (□), control left; (◇), diclofenac right; and (▲), diclofenac left. *No significant difference when compared with the baseline value (i.e. the mechanical allodynia is relieved) ($P < 0.05$). †Significant difference between control and diclofenac-treated animals ($P < 0.05$). #Significant difference compared with the baseline value observed on Day 1 AM on the non-inflamed side ($P < 0.05$). The arrows show the time points at which carrageenan was injected.

spread to the contralateral hind paw in 10% of the animals. Furthermore, the animals also displayed a loss of bodyweight and a persistent flexion of the knee joint. All of these signs suggest that Freund's adjuvant caused more massive inflammation and pain.

Several reports have demonstrated the histomorphometric changes after repeated carrageenan injection into a joint,[17,18] but only Daher and colleagues assessed the influence of repeated injections of carrageenan (72 h apart; 300 μg) into the knee joint on the mechanical nociceptive sensitivity by determining the paw elevation time.[6] The first injection caused relatively short-lasting nociception (at least up to 8 h), and 72 h later the paw elevation time response had fully returned to those of control values. Following a second injection 72 h after the first, the nociceptive responses evoked by subsequent inflammatory insults were significantly increased relative to those evoked in a naive joint. However, we observed some differences in our inflammatory model; that is, we did not find increased thermal and mechanical hyperalgesia after the second and third inductions of inflammation. This might be because of the difference in the applied tests (paw elevation time *vs* gait analysis and PWD tests). Alternatively, it might be because of the different routes of administration (knee joint *vs* ankle joint).

A number of studies have reported that gait changes in the arthritic rat can be used as an objective measure of pain and that temporal and spatial changes in gait observed in inflammatory hyperalgesia models might be owing to the avoidance of the normally non-noxious mechanical stimulation induced by walking.[19–21] Min *et al.* recently described a novel method that allows convenient measurement of the severity of arthritic pain in the knee joint in voluntarily walking rats.[10,22,23] Their results suggest that this is an effective tool with which we can determine arthritic pain under dynamic conditions. One of the advantages of their method is that the animals walk voluntarily and, therefore, stress imposed on the animals during the test is minimal. Furthermore, their method reflects painful states more realistically because it measures the weight load while the rat is walking. Accordingly, it determines primary hyperalgesia and it seems more sensitive than the PWD test, which assesses secondary thermal hyperalgesia. However, we have also detected some weaknesses in their model. We observed that the animals often stopped

in the corridor, making it difficult to determine the exact weight load for all four legs. Moreover, in contrast to the results of Min *et al.*, water deprivation did not constitute a strong drive for the rats to walk to the dark box. Hence, we applied a vanilla smell,[24] which seemed more appropriate. Our results revealed that the device might be an appropriate method for the long-lasting investigation of ankle joint pain.

The mechanisms of carrageenan-induced inflammation and subsequent hyperalgesia have been studied extensively.[25–27] The induction, maintenance and spread of chronic hyperalgesia could result from a series of peripheral and central changes occurring at the site of the insult and at spinal or supraspinal sites. Cyclooxygenase inhibitors, including diclofenac, are widely used therapeutic options in the treatment of inflammatory disorders, but their long-term usage also causes serious adverse effects, such as gastrointestinal haemorrhage.[28] It has been shown that oral treatment with 3 mg/kg diclofenac is effective against inflammation in rats,[7,8] and we therefore applied this dose in our experiments. In the present study, we found that this dosage exhibited different degrees of efficacy for the various parameters. Thus, anti-oedemic effects appeared earlier than anti-allodynic effects, whereas it did not influence secondary thermal hyperalgesia. The anti-oedemic potency of NSAID is a well-known effect, which is a result of the decrease in the level of prostaglandins.[29]

However, several others factors could cause pain, including nerve growth factor, which is not affected directly by diclofenac.[16] Thus, the increased rate of recovery of mechanical hyperalgesia during diclofenac treatment suggests that the anti-inflammatory effect of diclofenac caused a delayed decrease in mechanical pain. There is another possibility for its varying potency in the tests. The cross-sectional area indicates the enlargement of the joint and has a positive direction but the behavioural tests, including the PWD and the weight load tests, usually show that pain decreases. Consequently, the test results have a negative direction and might be saturated, thus this discrepancy may result from the saturation of pain behaviour. So, the behavioural test can be used for observing the slope of recovery from the maximal painful status. The slope of recovery seems to be fairly constant after each injection, and clearly shows the significant antihyperalgesic effect of diclofenac on mechanical hyperalgesia after the third injection. As secondary thermal hyperalgesia indicates central excitation, changes in the gait reflect mainly primary hyperalgesia. Thus, we suggest that this dose of diclofenac (i.e. 3 mg/kg) was not sufficient to influence central changes significantly.

In conclusion, we have presented a regional, limited form of repeatedly induced arthritis for long-term studies of inflammation and nociception without causing signs of a systemic disease. The findings of the present study clearly demonstrate the development of chronic hyperalgesia following repeated injections of carrageenan into the ankle joint of rats. Furthermore, with the repetition of carrageenan administration the time-course of the inflammation could be well controlled. Our experimental paradigm may provide a model that is suitable for the investigation of drugs or treatments that express their effects several days after their application. The advantage of this model is to extend the period of inflammation, and we suggest that by increasing the number of carrageenan injections, the inflammatory changes and the effects of treatment could be followed for an even longer time. Our present findings cannot be extrapolated directly to the clinical management of pain, but this experimental model of repeated acute joint inflammation may be a suitable model for painful joint disorders with recurrent flare-ups in humans (e.g. rheumatoid arthritis).

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