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Experimental study of the pharmacological modulation of nociception in an animal model of Osteoarthritis

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Assinatura: 

Experimental study of the pharmacological modulation of nociception in an animal model of Osteoarthritis

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Abstract

Pain in osteoarthritis (OA) is characterized by being present at rest but typically worsening with weight bearing and movement of the affected joint. Despite the high prevalence of pain associated with OA, it remains the least studied feature of this pathology, with the pharmacological control of OA-associated chronic pain being far from optimal. Previous studies have shown that the Knee-Bend and CatWalk tests are clinically relevant and useful to assess movement-induced nociception in the mono-iodoacetate (MIA) model of OA. The aim of this study was to investigate the effect of the administration of the opioid morphine (6 mg/Kg, subcutaneous), the non-steroidal anti-inflammatory drug (NSAID) diclofenac (30 mg/Kg per os) and the local anaesthetic lidocaine (5 mg, 10% solution, intra-articular) in the nociceptive behavior assessed by those two tests in control and OA animals.

The effect of drug administration on nociceptive behavior was evaluated at days 3, 20 and 31 post MIA administration. The three drugs significantly reduced the Knee Bend score at all time points, although the diclofenac effect at day 20 was much less pronounced. On the CatWalk test, all drugs showed an increase on the ipsilateral paw intensity at day 3, but at day 20 only morphine showed to be significantly effective.

These results further validate the use of the Knee-Bend and CatWalk tests in the evaluation of movement-induced nociception, showing their potential usefulness in the study of the analgesic efficacy of drugs in OA treatment.

Keywords: Osteoarthritis; Nociception; Pain; CatWalk; Knee-Bend; Pharmacology

Introduction

Osteoarthritis (OA) is the most common type of articular disorders [1,14]. It involves the whole joint and is characterized by focal areas of damage in the articular cartilage, sclerosis of the subchondral bone, outgrowth of osteophytes and variable synovitis and capsular thickening [9,14]. Pain worsened by weight bearing and movement of the affected joint is one of the cardinal symptoms of OA. It is responsible for the high rate of disability and quality of life impairment, especially in the elderly, due to functional limitations [5,16].

Articular cartilage has a striking role in the pathogenesis of OA but, since it is not innervated, it cannot be the tissue that directly generates pain. By contrast, there are suggestions that the afferent innervation of subchondral bone, periosteum, synovium, ligaments and the joint capsule could be the source of nociception [7,9,11]. In the lack of disease-modifying therapeutic actions, pain management becomes a critical cornerstone of symptomatic OA treatment, contributing to the improvement of joint mobility and reduction of functional impairment. Unfortunately, even the pharmacological control of OA-associated chronic pain is far from optimal with the current pharmacological approaches (including non-steroidal anti-inflammatory drugs (NSAIDs) and opioid formulations) not providing the adequate relief for this condition [5,6,14].

Several experimental animal models have been developed to mimic human OA. The intra-articular injection of monosodium iodoacetate (MIA) in the rat knee has been described as the best model for studying OA pain [10,11,17,25]. MIA is a glycolysis inhibitor that disturbs the condrocyte metabolism, inducing progressive cartilage degeneration and histopathological and behavioral changes similar to the human disease [13].

Ferreira-Gomes *et al* [11] described the CatWalk and Knee-Bend assays as feasible methods to measure joint-related pain in the MIA model. These tests allow a clinically relevant evaluation of weight-bearing and movement-related pain. To better validate the usefulness of these tests, we performed a study on the pharmacological modulation of nociception in this model, using three analgesic drugs from different classes: a non-selective NSAID, diclofenac, the opioid morphine, and lidocaine, a blocker of sodium channels commonly used as a local anesthetic. Doses were chosen based on data retrieved from other animal models of OA [10].

As previously shown in this model [2,6], at day 3 there is an inflammatory state that seems to be resolved by day 7. After day 14 a sustained state of pain is established [6], therefore, we evaluate the drug effect on the behavioral tests at day 3, an early and inflammatory stage of

the disease, and at day 20, when the disease is fully established, presenting the typical histological changes.

In this study, by addressing the question of how the analgesic drugs administration affect noxious-stimuli evoked behavioral response we try to better evaluate the usefulness of the CatWalk and Knee-Bend test as clinically relevant tools for pain evaluation in an animal model of OA

Materials and Methods

Experimental animals

Adult male Wistar rats (Charles River, Lyon, France) weighing 250 ± 50 g at the time of the knee injection (day 0) were used in these experiments. Animals were housed at a maximum of three per solid-bottom cage, with water and food *ad libitum*, and the animal room temperature was kept at constant temperature of 22º C on a 12-hour light/12-hour dark cycle. Adequate measures were taken to minimize pain or discomfort of the animals, and all experimental procedures were performed in accordance with the ethical guidelines for the study of pain in conscious animals, [28] as well as the European Communities Council Directive 86/609/EEC.

Induction of Osteoarthritis

Under brief isoflurane anaesthesia, animals were injected with the use of a Hamilton syringe (Hamilton, Reno, NV) inserted through the patellar ligament into the joint space of the left knee, with 25 µL of either saline or 2 mg of MIA in saline (Sigma-Aldrich, St. Louis, MO). Contralateral knees did not receive any treatment.

Behavioral Testing

Animals were habituated/adapted to the experimenter and to the testing situation for at least 1 week before the start of the experiment and 5 to 10 minutes before each testing, until exploration activities ceased. For each rat, Knee-Bend and CatWalk testing was applied before the knee injection (day 0) to assess the baseline response of each animal and 3, 20 and 31 days after the injection. All tests were done bilaterally.

Knee-Bend Test

To evaluate knee movement nociception, a variation of the ankle-bend test of nociception for monoarthritic rats was performed [18]. For this purpose, animal movements were

restricted while preserving access to both hind limbs. The test consists of 5 flexions and extensions of the knee joint, performed by the experimenter, with the recording of the number of vocalizations and/or struggle reactions in response to movement. The test score is determined according to the type of the reaction and manipulation that instigated that reaction following this evaluation scale: Score 0 is given to no response to any kind of flexion/extension of the joint; score 0.5 when struggle occurs to maximal manipulation; score 1 when struggle occurs to moderate and also when squeak reaction occurs to maximal manipulation; and score 2 is given to vocalization in response to moderate flexion/extension of the knee joint. The sum of the reactions, giving the maximal value of 20, represents the knee-bend score, an indication of animal's nociception.

CatWalk

The CatWalk test measures the total intensity of the contact area of each paw, becoming a tool to evaluate animal disability and nociception. Animals were allowed to walk freely, over a glass platform in a dark compartment, while a light beam illuminated the platform in a way that light was reflected downward only at the points where the paw touched the glass surface, providing us an image of the paw print. The animal behavior was monitored by a video camera placed under the glass platform and connected to a computer equipped with video acquisition software (Ulead Video Studio, Freemont, CA). The intensity is dependent on the area of the paw in contact with the platform and the pressure applied by the paw. The higher the pressure applied and the paw contact area, the higher the intensity of the signal. Six random frames of the videos recorded during rat evaluation, three with the animal walking and three with the animal standing still, were analyzed using ImageJ 1.37 (available at www.tucows.com/preview/510562). The number and intensity of pixels above a defined threshold were quantified. Results are expressed as the percentage of the ipsilateral hind paw print over the total intensity of both hind paws.

Pharmacological Evaluation

Sodium diclofenac (Sigma-Aldrich, St. Louis, MO) and morphine (Labesfal, Portugal) were dissolved in double-distilled water and saline respectively. Lidocaine (Sigma-Aldrich, St. Louis, MO) was administered as a 10% saline solution. After baseline testing, drugs were administered to OA rats either orally (p.o; diclofenac, 30 mg/Kg, n=5), subcutaneously (s.c; morphine, 6 mg/Kg, n=5) or intra-articularly (i.a; Lidocaine, 5mg, n=5 or saline, n=5), 3, 20 and 31 days after OA induction. Lidocaine effect was also tested in control animals at the same dose (n=5). At days 3 and 20 of disease progression, behavioral responses were assessed 10,

20 and 30 minutes after lidocaine's intra-articular injection. Morphine's effect was evaluated 30, 60, 90, 120 and 180 minutes after the drug administration and diclofenac's at 30, 60, 90 and 120 minutes after the treatment. At 31 days, behavioral response was only evaluated at drug's maximum effect time – 10 minutes for lidocaine, 60 minutes for morphine and 30 minutes for diclofenac as shown by days 3 and 20 results.

Tissue Processing

At 31 days, 2 hours after Knee-Bend testing, the rats were deeply anesthetized with Chloral Hydrate (35%, i.p.) and sacrificed by intracardiac perfusion with 250 mL of Tyrode's solution and 1 L of a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB). After perfusion the knee joints were dissected, postfixed for 72 hours in the same fixative solution and then decalcified for 8 hours with a decalcification solution containing 7% $AlCl_3$; 5% formic acid and 8.5% HCl. After this, the joint was washed in 0.1 M PB and cryoprotected in a 30% sucrose solution in 0.1 M phosphate buffer saline (PBS) with 0.01% sodium azide until they were cut into 20-µm sections using a cryostat. Knee sections were stained by Fast Green and Safranin-O method. They were mounted with Eukitt (Kindler, Freiburg, Germany) and photographed with the use of a Nikon Eclipse E200 microscope with a DFK 41F02 color digital camera (Imaging Source, Bremen, Germany) attached.

Data analysis

Data is expressed as mean ± SEM. The comparison between ipsi and contralateral knees was analyzed using a paired t-test. Disease progression (time-course) and drug response was compared using repeated-measures ANOVA. Statistical significance was considered at the p value inferior to 0.05.

Results

Histological analysis of the knee joint

Joint histology was evaluated in all animals at day 31 through Fast-Green and Safranin-O stained sections (Fig. 1). The observed changes were similar to those previously described [24].

In MIA injected animals, it was observed a decrease in the articular cartilage thickness due to chondrocytes and matrix proteoglycan's loss, as indicated by the marked reduction of Safranin-O stain. There was also exposure of subchondral bone and subchondral bone thickening. These alterations were similar and confirmed in all OA cases. Control animals showed no alterations in cartilage or subchondral bone structure.

Behavioral Testing

Control animals show no nociceptive response when evaluated by Knee-Bend and CatWalk tests.

The injection of MIA into the left knee induced a significant increase in the Knee-Bend score of the ipsilateral knee verified at day 3, which was maintained throughout the study (Fig. 2). In OA animals, the ipsilateral paw-print intensity, as assessed by the CatWalk test, was significantly decreased from day 3 until day 31 (Fig. 3).

Pharmacological evaluation

Lidocaine

The effect of intra-articular administration of lidocaine on OA-induced nociception was evaluated at 10, 20 and 30 minutes after its injection (Fig. 4). At day 3, 10 minutes after lidocaine injection there was a significant reduction of the knee-bend score of the affected knee from 11,2 ± 1,5 to 1,2 ± 0,7 ($p<0.01$). Afterwards, the effect starts to diminish and at 30 minutes after the administration it is completely reverted to baseline levels (Fig 4A). This was also verified at day 20 ($p<0.01$) and at day 31 ($p<0.05$).

Lidocaine effect on CatWalk at day 3 was also observed 10 minutes after the drug injection with an increase from 25,2 ± 4,8 to 38,2 ± 4,4 ($p<0.05$) immediately reversing at 20 minutes (24,9 ± 2,9). At days 20 and 31, although there was a similar trend, no significance was observed (Fig. 4B).

Lidocaine did not produce any effect on the control animals (data not shown).

Morphine

Morphine's Knee-Bend ipsilateral response was observed 30 minutes after drug administration at both days 3 and 20. There was a reduction from 11,4 ± 0,7 to 0,0 ± 0,0 at day 3 ($p<0.001$) and from 11,0 ± 1,1 to 0,6 ± 0,4 at day 20 ($p<0.001$). This effect is still observed at 90 minutes, and starts to revert at 120 minutes after morphine administration (Fig. 5A). A score reduction is observed 60 minutes after drug administration at 31 days ($p<0.01$) to near-zero levels (0,6 ± 0,4).

Morphine effect on CatWalk score at days 3 and 20 starts at 30 minutes after drug administration but only reaches significant values at 60 minutes ($p<0.05$ at day 3 and $p<0.001$

at day 20). Drug effect starts to revert at 120 minutes (Fig. 5B). At 31 days, there was a significant increase on left-hindpaw total intensity 60 minutes after drug administration ($p<0.05$).

Diclofenac

Diclofenac maximum effect is observed at 30 minutes post drug administration, with ipsilateral Knee-Bend scores at day 3 significantly reducing from 14,6 ± 0,9 to 2,0 ± 0,4 ($p<0.001$; Fig. 6A). Complete drug effect reversion is noted at 120 minutes. At 20 days there was a significant decrease 30 minutes after drug administration ($p<0.05$) reversing at 120 minutes (10,6 ± 1,8 before drug injection to 6,2 ± 1,1, 30 minutes later). Nevertheless the effect was much less pronounced than at day 3 (Fig. 6A). No effects of diclofenac on Knee-Bend score were noted at day 31.

At day 3, 30 minutes after diclofenac administration there is an increase in CatWalk score from 22,6 ± 4,0 to 37,8 ± 6,2 ($p<0.01$). At 20 or 31 days, diclofenac administration didn't show any effect on the CatWalk test (Fig. 6B)

Discussion

Chronic pain is usually accompanied by an increased response to painful stimuli, hyperalgesia, and innocuous stimuli, allodynia. Such hypersensitivity could be related to peripheral pain sensitization, mediated by cytokines, and/or central pain sensitization at subcortical or cortical level, already described in the pathogenesis of OA [7]. Various behavioral tools have been used to evaluate OA-related pain in the MIA model [2,5,17], however none of them has been validated as a significant mean to study damaged tissues-related (primary) hyperalgesia, as most of them focus on secondary hyperalgesia and secondary allodynia. With the pharmacological study here described in this model during 31 days, we try to validate and better understand the usefulness of Knee-Bend and Catwalk to evaluate knee-joint movement-elicited nociception, a common trait of OA [12].

Morphine is an opioid with strong analgesic properties that exerts its effects mostly at CNS [26]. Its analgesic effect is mainly due to its activation of the inhibitory mu-opioid receptor located both at spinal cord and supraspinal sites. This activation leads to reduced transmitter release from nociceptive afferent fibers as well as an attenuation of neuronal processing of pain messages [26]. In our study, morphine provided a remission of Knee-Bend score to nearly zero-values in ipsilateral knee, due to its efficacy as an analgesic drug (Fig. 5A). The increase in

ipsilateral paw contribution to total weight bearing could easily be compared to that of non-OA animals, supporting the nociceptive pathways block promoted by this drug (Fig. 5A). This morphine nociception reversing effect, provide a support to the Knee-Bend testing and to the CatWalk approach as valid methods to measure MIA-induced OA-related pain. Morphine-related ipsilateral knee reduction on nociception can contribute to the reduction of contralateral nociceptive response. In humans, the only published study on morphine efficacy revealed improvement in the overall quality of sleep and reduced pain in patients with moderate-to-severe OA pain [4]. This, as other preclinical studies, favor clinical research on opioids role in OA pain.

Pain presents direct afferent, intrinsic regulatory and higher symbolism, associating cortical components with many biological and psychological regulating elements. Morphine efficacy in this model may result, not only, from reduced sensitized peripheral neurons activation of central neurons but also from its interaction with descending control pathways. Morphine psychoactive effects (in prefrontal opioid pathways) could add an increase to the ipsilateral paw contribution to the weight-bearing activity. Detailed comprehension of morphine-acting pathways would be very useful in the OA-related pain preclinical study as for the management of OA patients.

Lidocaine exerts its effects by blocking the fast voltage-gated sodium (Na^+) channels in the neuronal membrane [3]. The excitable membrane of nerve axons maintains a resting transmembrane potential of -90 to -60 mV. During excitation, the sodium channels open and a fast inward sodium current quickly depolarizes the membrane. As a result of depolarization, the sodium channels close and the potassium channels open [21]. The outward potassium current repolarizes the membrane. With sufficient blockade, the membrane of the postsynaptic neuron will not depolarize and so it fails to transmit the action potential leading to lidocaine anesthetic effects. Lidocaine effectively reverted the nociception-related behavior as assessed by the significant reduction in the Knee-Bend score (Fig. 4A). This is consistent with the blockage of signal transmission to central structures, reflecting this test significance in the evaluation of primary hyperalgesia.

CatWalk provides an indirect weight-load analysis in the context of movement related-nociception study. In human unilateral knee OA it was observed an increase in contralateral leg load [7], so, weight-load analysis can reflect the significance of OA pain in human daily life activities. Facing that, OA-patients gait depends not only on the primary joint hyperalgesia but, also, on the combination of that movement restricting factor with complex neural interacting

pathways, some of them with supraspinal origin. A change in one of the locomotion modulator stimuli (as the nociceptive stimuli removal) implies the reorganization of the march related pathways and the "learning" of a new way of walk. This learning process is certainly time-dependent and is necessary a significant time period to allow the habituation to the new non-painful condition and, with that, the development of a new "walking way" with a statistically significant difference. The quick acting lidocaine, with its effect peaking at 10 minutes and reverting 20 to 30 minutes after administration, does not provide the necessary time period to the occurrence of this adaptation process, which may explain the lack of contribution of ipsilateral hindpaw weightbearing at days 20 and 31. In contrast, morphine effect is way more sustained (felt from 30 to 120 minutes) and this longer time period allows the rat to adapt to its new "non-nociceptive" situation, resulting in the significant detection of new weight-load pattern 60 minutes after drug administration.

Diclofenac, as other non-steroidal anti-inflammatory drugs (NSAIDs), is a common therapeutic option in the management of middle to moderate OA pain. During the progression of the inflammatory process, many prostanoid products (Prostaglandin E2, D2, F2, I2 and thromboxane) are synthesized by the action of cyclo-oxygenase (COX) enzymes, thereby, the blockage of the enzymes COX-1 and COX-2 is helpful in the management of an inflammatory pain situation [8,15,27]. Pulichino and colleagues [20] documented, following MIA injection, a rise in prostaglandins levels that return to baseline by day 7. Histological analysis also demonstrates the presence of early inflammation in the MIA model and the subsequent resolution of the inflammation by day 7 [2,6,10]. In the present study, diclofenac was highly effective at day 3, in a period of disease progression characterized by the inflammatory state. The absence of inflammation at days 20 or 31 may explain the decrease of efficacy of diclofenac in this model at these time points. A slight significant decrease in Knee-Bend score at day 20 thirty-minutes testing, was observed but was not accompanied by any CatWalk increase in ipsilateral hindpaw contribution increase. This helps to illustrate the difference between these tests properties, the Knee-Bend has most likely a higher sensitivity perceiving primary hyperalgesia comparing to CatWalk (Fig. 6). The lack of relevant diclofenac's effect at days 20 or 31 argues in favor of the lack of inflammatory mechanisms in OA pain at later time points.

Previous studies [5,10] tried to assess acute diclofenac's antinociceptive effect on various behavioral endpoint and we can find some discordant results. Fernihough et al. [10] have noticed a significant secondary mechanical hyperalgesia reduction with diclofenac at day 3 but none at days 14 or 28; this is similar to our primary hyperalgesia reduction pattern. Twenty-

one days Pomonis et al. [17] results showed no significant change in weight bearing with the administration of celecoxib or indomethacin. These results contrast with Chandran and collegues [5], that, using the grip-force test, observed, 20 days after 3 mg MIA injection, an antinociceptive effect attributable to 30 mg/Kg (100μmol/Kg) doses of diclofenac. Ivanavicius et al. [12], aware of these works used weight-bearing methods and proved celecoxib inefficiency after day 14; remarkably he found significant naproxene antinociceptive effects at day 14. These authors used different MIA doses ranging from 1 to 3 mg and there seems to be no trendline relating doses, disease progression and its dependent response to NSAIDs. Further studies are necessary to understand the role of different clinical relevant NSAIDs in the early inflammatory pain state observed in the MIA-induced model and a standardized approach with the Knee-Bend and CatWalk tests would provide a deeper insight in this subject.

The study of the pharmacological modulation of a known pain-related molecular marker would provide a powerful support to the validation of any pain-related behavioral study. Noxious stimuli have been shown to induce Fos expression in sub-populations of spinal cord neurons located predominatly in laminae I, IIo and V [20]. Despite low Fos specificity for these stimuli, previous works [19,23] showed that pre-treatment with morphine prevent spinal Fos activation. Studies to evaluate different drug-induced Fos pattern changes in this model are already in progress.

The pain behavior associated with intra-articular MIA injection is biphasic in nature with an early inflammatory phase that lasts until day 7 [6] and a second, chronic phase beginning at day 14. Bove et al. [2] showed that bone damage only appears after day 7. The delayed onset in human OA pain suggest that, even fronting the scarcity of knowledge regarding OA-nociceptive mechanisms, a large part of pain afferents could come from subchondral bone. In this model, the study of the efficacy of therapeutically relevant agents becomes clinically significant when done at later time points such as 20 days after MIA injection. In this study we provide compelling evidence of the high sensitivity and strength of the CatWalk and the Knee-Bend tests to evaluate movement-induced nociception. The different antinociceptive profiles of morphine, lidocaine and diclofenac, deeply linked and in agreement to their different OA-associated pain reversal mechanisms, provides useful information about these tests' value in the study of the analgesic efficacy of drugs in OA treatment.

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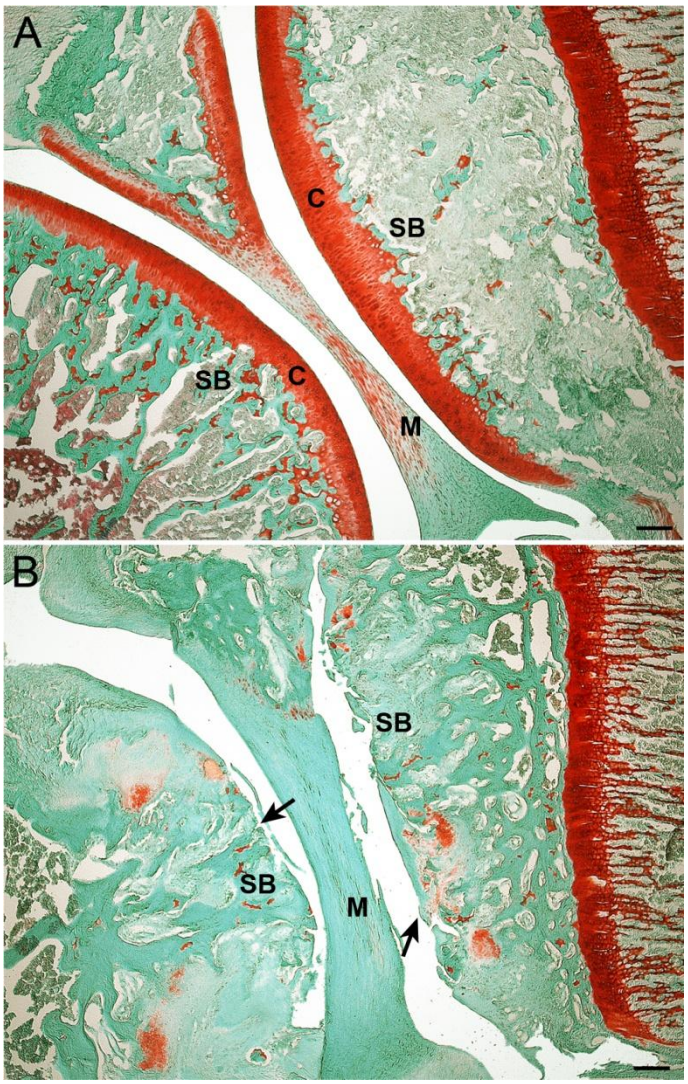

Fig. 1 – Fast-Green and Safranin-O stainig of knee joint 31 days after saline (A) or 2 mg MIA (B) injection. MIA injected animals show destruction of articular cartilage (C) with subchondral bone (SB) changes (arrows). M: Menisci.

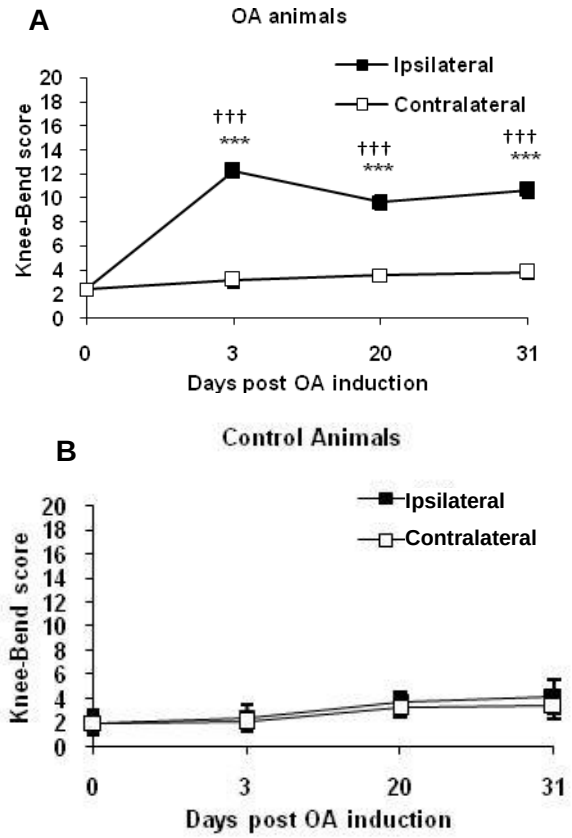

Fig. 2 – Time course changes in Knee-Bend score in MIA (A, n = 20) and saline (B, n = 5) injected rats (mean ± S.E.M.). Baseline scores were determined in both knees for all animals before injection (day 0). *** P < .001, significantly different from day 0 levels (repeated measures ANOVA). †††P < .001, significantly different from contralateral knee (paired t-test). No difference is observed, during disease progression time, between ipsi and contralateral paw in saline injected animals (B).

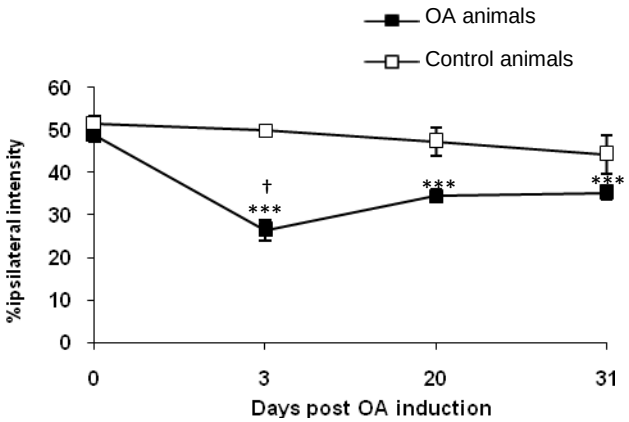

Fig. 3 – Time course changes in the percentage of the ipsilateral paw print over total intensity assessed by the CatWalk test. Paw print intensity baseline scores were determined for all animals before injection (day 0). *** $P < .001$, significantly different from baseline levels (repeated measures ANOVA). †$P < .05$, significantly different from control animals (1-way ANOVA). No difference is observed, during disease progression time, between ipsi and contralateral paw in saline injected animals (B).

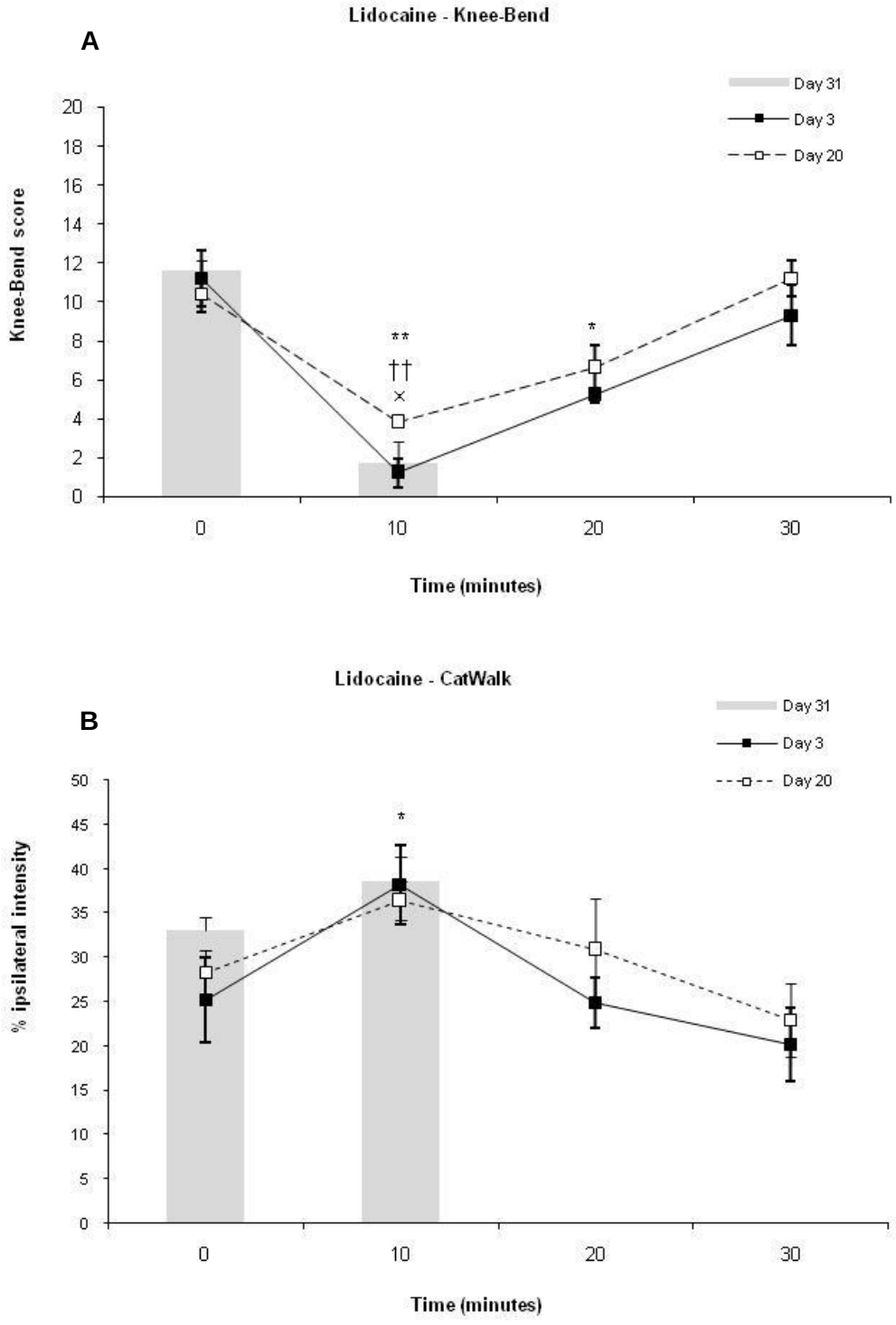

Fig. 4 – Effect of lidocaine on MIA-injected rats assessed by Knee-Bend (A) and CatWalk (B) tests on days 3, 20 and 31. Day 31 results were plotted as a bar graph. * and ** represent significance levels of $p < .05$, and $p < .01$ at day 3. †† represent significance levels of $p < .01$ at day 20. x represents significance levels of $p < .05$ at day 31. Effect on Knee-Bend test is verified both at days 3, 20 and 31. CatWalk-assessed reduction on nociception is only verified at day 3.

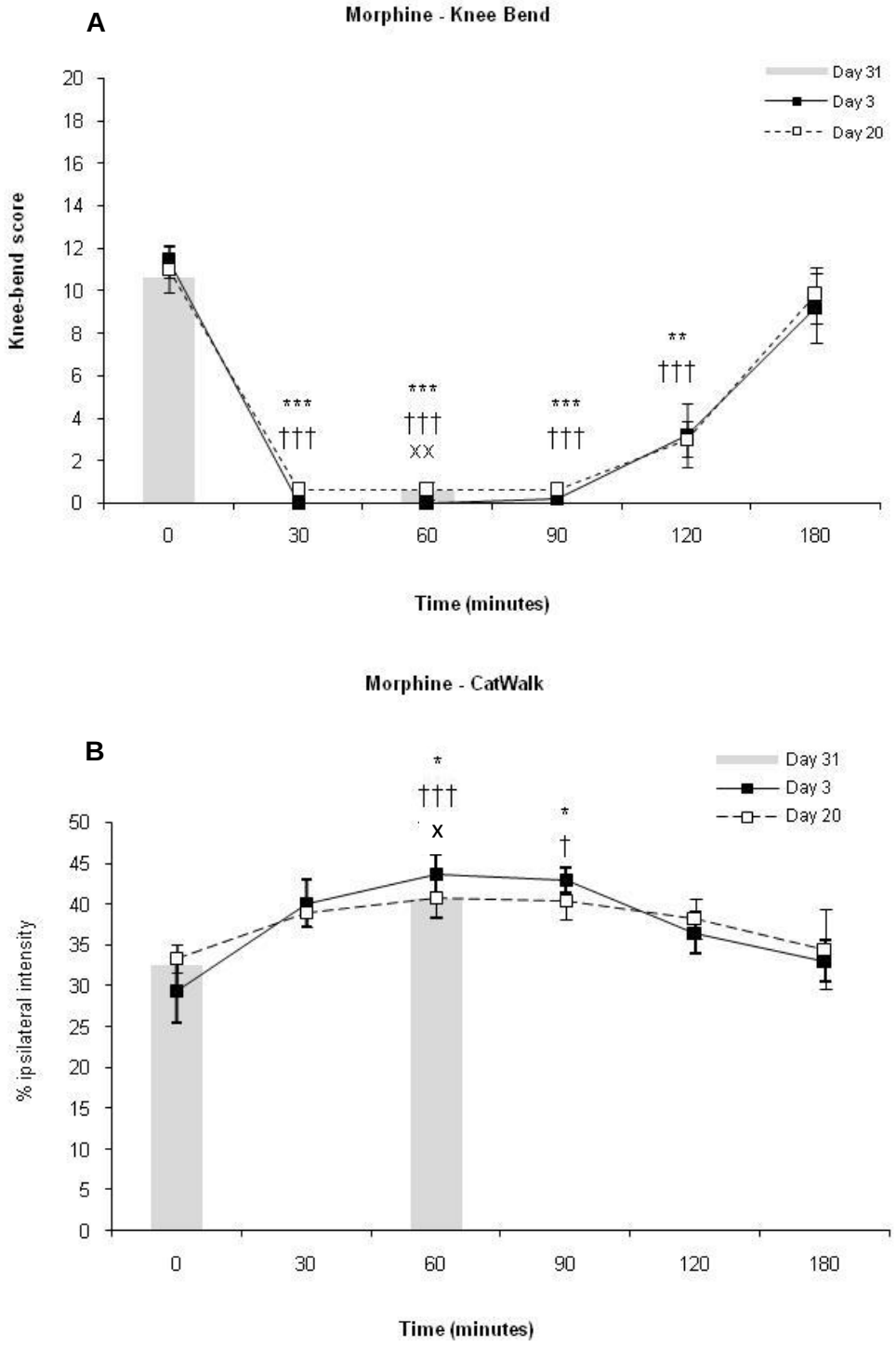

Fig. 5 – Effect of morphine on MIA-injected rats assessed by Knee-Bend (A) and CatWalk (B) tests on days 3, 20 and 31. Day 31 results were plotted as a bar graph. *, ** and *** represent significance levels of $p < .05$, $p < .01$ and $p < .001$ at day 3. † and ††† represent significance levels of $p < .05$ and $p < .001$ at day 20. x and xx represents significance levels of $p < .05$ and $p < .01$ at day 31. Morphine nociception reduction, as evaluated by Knee-Bend test, is observed at 3, 20 and 31 days and already detected 30 minutes after administration. CatWalk test detect significant nociception reduction at days 3, 20 and 31.

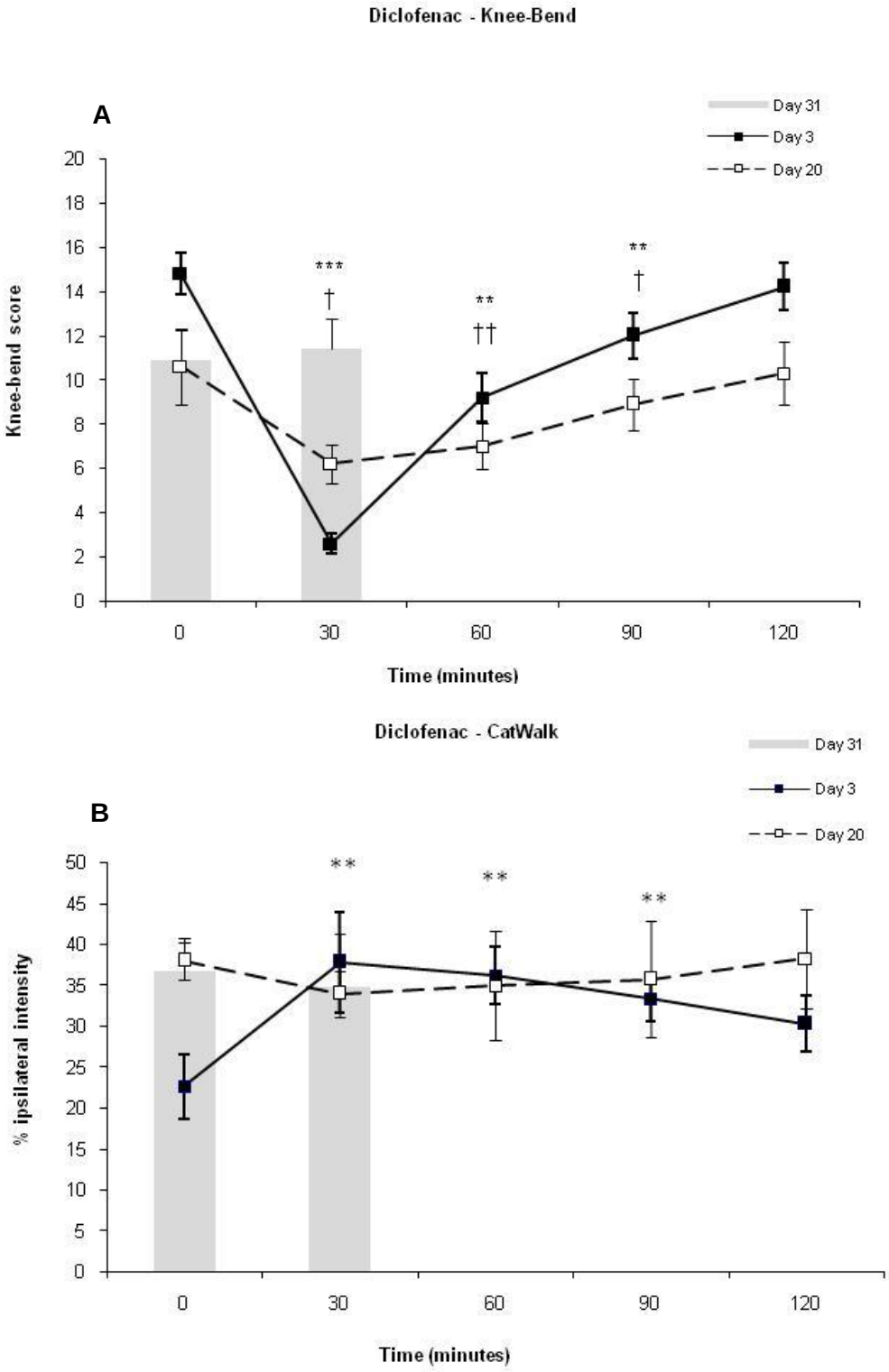

Fig. 6 – Effect of diclofenac on MIA-injected rats assessed by Knee-Bend (A) and CatWalk (B) tests on days 3, 20 and 31. Day 31 results were plotted as a bar graph. ** and *** represent significance levels of $p < .01$ and $p < .001$ at day 3. † and †† represent significance levels of $p < .05$ and $p < .01$ at day 20. At day 3, significant changes are noted on CatWalk and Knee-Bend tests. At day 20 changes are only observed on Knee-Bend tests. No significant changes were found at day 31.