

Research Article

Development and characterisation of a novel animal model of prostate inflammation-induced chronic pelvic pain

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Abstract. Chronic pelvic pain due to prostate inflammation is a significant clinical problem. In the current study we developed and validated an animal model of inflammation-induced pelvic pain (NIH category IIIA). 3% carrageenan was injected into the ventral prostate in SD rats. At different time points (before and after 48 h, 72 h and 1 wk of injection), radiant heat and von Frey filaments (mechanical stimuli) were applied to different pelvic areas. The escape latency (s) from radiant heat, and the bending force (g) of the filament to which the animal responded by moving were taken as measures of heat and mechanical thresholds respectively. Inflamed animals showed a significant reduction in mechanical threshold (mechanical allodynia) at 72 h and 1 wk, and a significant reduction in heat threshold (thermal hyperalgesia) in the scrotal skin, compared to sham. Morphine (5 mg/kg., i. p.) significantly reduced both heat hyperalgesia and mechanical allodynia. It is expected that this novel model will prove to be useful in studying the neurobiological mechanisms of male pelvic pain.

Key words: Prostate – Prostatitis – Chronic pelvic pain – Inflammation – Hyperalgesia – Morphine – Carrageenan – Male

Introduction

Prostatitis is often a painful condition, prevalent in 4–14% of the general population (Mehik et al., 2003). It is estimated that 50% of men suffer from symptoms of prostatitis at some point in their lives (Stamey, 1980). Of these, more than 90% of the patients suffer from chronic prostatitis/chronic pelvic pain

syndrome (CP/CPPS), a chronic painful condition classified as category III prostatitis under the NIH consensus classification, where bacterial infection is absent (Krieger et al., 1999; Nickel, 2003). Category III prostatitis is classified into sub-categories IIIA and IIIB, depending on the presence or absence respectively of inflammation of the prostate (Nickel, 2003), although this classification is controversial (Pontari, 2003). Chronic pelvic pain, testicular pain, and pain or discomfort during voiding and/or ejaculation are some of the common and debilitating symptoms of category III chronic prostatitis (Nickel et al., 2001; Shoskes et al., 2004; NIH-CPSI), chronic pelvic pain being the most disturbing symptom (Krieger et al., 1999). In the NIH-Chronic Prostatitis Symptom Index (NIH-CPSI), pain in the pelvic area, penis and testicles, and pain on urination and ejaculation are considered as major symptoms of chronic prostatitis, along with urinary symptoms and reduction in quality of life (NIH-CPSI).

Despite the high prevalence and lack of optimal treatment, the mechanism of pain development in prostatitis is poorly understood. This is partly due to the lack of a suitable preclinical model of prostatitis-induced pain or prostaticodynia. There are several animal models of prostatitis developed in the past, mainly studying the basic histopathology and inflammatory mechanisms (Ishigooka et al., 2000; Lang et al., 2000; Rivero et al., 2002; Diserio and Nowotny, 2003; Gilleran et al., 2003; Wilson et al., 2004). Some of these models used chemical irritants such as capsaicin, ethanol or dinitrobenzenesulfonic acid, which could cause tissue destruction (Lang et al., 2000). A human study has investigated the neurophysiology of chronic pelvic pain syndrome (Lee et al., 2001). However, an animal model specifically characterising the pain behaviour caused by inflammatory prostatitis has not been reported in the literature. An animal model which can mimic prostatitis-induced chronic pelvic pain will help

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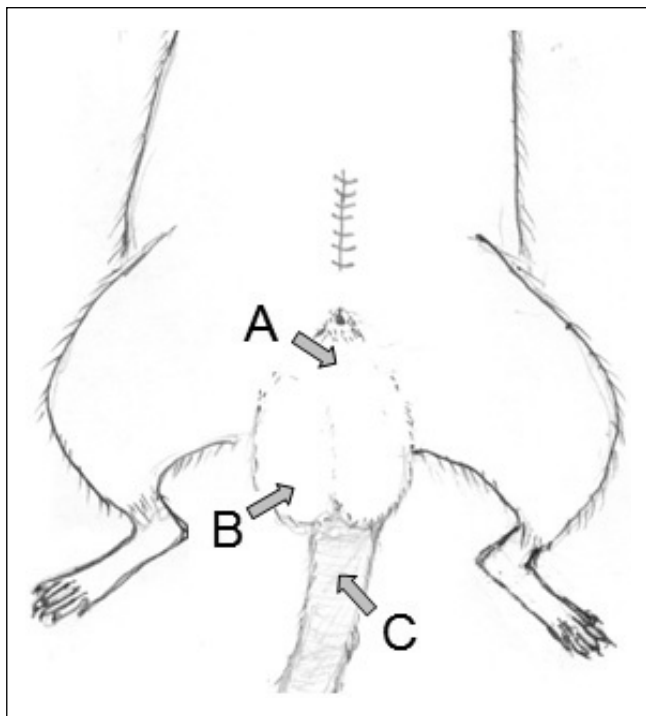


Fig. 1. Shows the pelvic areas where the heat and mechanical stimuli were applied in the rat. Reductions in heat and mechanical thresholds were observed in the skin overlying the scrotum (area B), but not in areas A and C.

to study the molecular mechanisms of pain sensation, transduction, and pain processing at different levels, in this condition. A preclinical model will also help to study the central changes occurring which are probably responsible for the chronicity of pelvic pain.

Injection of high concentrations of carrageenan has been shown to cause chronic pain of muscle tissues. Therefore, the current proposal aims at developing and characterising an animal model of chronic pelvic pain by inflaming the prostate gland with aseptic injection of sterile carrageenan, thus avoiding bacterial infection of the prostate. This model could mimic the NIH category IIIA prostatitis, where inflammation, and not bacterial infection, is believed to be the major cause of pelvic pain. Carrageenan is a polysaccharide which is very commonly used to induce inflammation and subsequent pain in various inflammatory pain models (Moncada et al., 1975; Eisenach and Gebhart, 1995; Kehl et al., 2000; Yaksh et al., 2006). We have previously shown that an injection of 3% carrageenan into the gastrocnemius muscle causes chronic reductions in threshold to heat stimulus (heat hyperalgesia) and mechanical stimulus (mechanical allodynia) in rats, whereas 1% carrageenan causes acute heat hyperalgesia and mechanical allodynia (Radhakrishnan et al., 2003). Therefore in the current proposal, 3% carrageenan will be used to induce inflammation of the prostate which is expected to produce chronic heat hyperalgesia and mechanical allodynia of the pelvic area. Carrageenan has the advantage that it causes inflammation without producing excessive tissue damage (Radhakrishnan et al., 2003) compared to chemical irritants used in some of the earlier models of

prostatitis (Lang et al., 2000). This model will better mimic the human non-bacterial prostate inflammation where tissue necrosis or damage is minimal. The current model is expected to be a valuable preclinical tool to study the neurobiology of prostate-related pain. Long term goal of this research is to identify major peripheral and central mechanisms of chronic prostatic pain (prostatodynia) due to different etiology.

Materials & Methods

The specific aims of this study are i) to induce prostate inflammation in rats by injecting carrageenan; ii) to assess changes in pain thresholds to heat stimulus, and to mechanical stimulus in various pelvic areas of inflamed animals (Fig. 1); iii) to validate this pain model using morphine, which is the gold standard analgesic. A reduction in heat threshold in inflamed animals compared to controls will indicate secondary heat hyperalgesia, and a reduction in mechanical threshold will indicate secondary mechanical allodynia, both of which are accepted as objective measures of pain.

i) Animals

Male Sprague-Dawley rats weighing 250–350 g ($n = 28$) were used for the experiments. Animals were kept in ambient conditions with free access to food and water in a 12h dark-light cycle. All experiments were approved by the Animal Care and Use Committee at the Western University of Health Sciences, and were carried out according to the guidelines of the International Association for the Study of Pain and National Institute of Health (Zimmerman, 1983).

ii) Drugs

Morphine sulphate and type IV λ -carrageenan were obtained from Sigma-Aldrich, MO, USA. Morphine was dissolved in 0.9% sterile saline. Sterile carrageenan suspension (3% w/v) was prepared by mixing heat-sterilised lambda-carrageenan powder in sterile normal saline in a laminar-flow hood.

iii) Induction of Inflammation

Thresholds to heat and mechanical stimuli were measured in two groups ($n = 6$ each) of SD rats as described below under 'Behaviour Pain Measurement'. Rats were anaesthetised using 5% isoflurane and maintained at 2–3% v/v isoflurane, keeping the animals on a water-circulating heating pad. The surgical area (lower abdomen above the penis), and the scrotal skin were shaved (for ease of applying heat and mechanical stimuli) and the skin in the surgical area sterilised using 3 applications of 70% v/v ethanol followed by 10% povidone-iodine solution. A 2% lidocaine solution was applied to the cleaned skin to reduce post-surgical pain and to reduce sensitisation of the areas adjacent to the wound due to surgery, which could affect future pain behaviour testing in those areas. After 10 minutes of lidocaine application, an incision (1 inch) was made in the skin and abdominal wall to expose the ventral lobes of the prostate gland (Jesik et al., 1982). Lidocaine solution was applied again to the wound. A sterile suspension of 3% carrageenan in a volume of 25 μ l each was injected in one group of rats (inflamed group, $n = 6$) into both right and left ventral lobes of the prostate gland using a disposable, sterile 30 G needle and a sterilised Hamilton syringe. The wound on the muscle layer was closed using #4.0 sterilised absorbable suture material and the skin wound was closed using sterile stainless steel wound clips with the aid of a clip applicator. The surgical procedure was carried out in a clean room, and every precaution was taken to avoid microbial infection of the prostate and the wound. Topical neomycin (antibiotic)

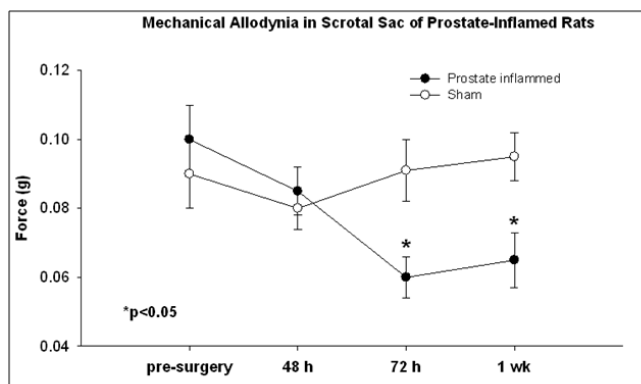


Fig. 2. Injection of 3% carrageenan into the rat prostate significantly reduced the threshold to mechanical stimuli (von Frey filaments) applied to the scrotal skin, at 72 h and 1 wk after the injection. *significantly different from non-inflamed sham animals ($p < 0.05$).

cream was applied to the clipped wound to enhance healing and thus to minimise any sensitisation of the nearby areas to pain testing. However, no systemic analgesics were administered before or after surgery, which could have affected the expected acute/chronic pain behaviour in this model. The decision not to use analgesics in this proposal was approved by the IACUC of Western University. In the sham group ($n = 6$) the same surgical procedures were followed and 25 μ l of sterile normal saline, instead of carrageenan, was injected into both the right and left ventral prostate lobes. All animals were allowed to recover in individual clean cages for 48 h. After 48 h of injection, animals were brought to the laboratory and acclimatised for about 1 hour. To assess the pain after inflammation, thresholds to heat and mechanical stimuli were measured in the inflamed group and the sham controls at 48 h, 72 h and 1 wk after injection, as described below.

iv) Behavioural Pain Measurement

Measurement of threshold to heat stimulus (to assess thermal hyperalgesia): Hargreave's method was used to measure thresholds to heat stimulus before and at 48 h, 72 h and 1 wk after carrageenan or saline injection in the inflamed and sham groups. The rats in both groups (inflamed and sham) were kept in Plexiglas[®] cubicles on the glass platform of a plantar analgesia testing equipment (IITC Life Science, CA) for 30 minutes to acclimatise. Radiant heat from a light source was shone on to the skin between the penis and the scrotum (Fig. 1, A), simultaneously starting a stop watch. When the animal moved from the original position to escape from the heat stimulus, the stop watch was stopped and the time duration was noted. The latency in seconds from the start of heat application till the animal's escape from the heat stimulus was taken as escape latency. The testing was repeated 3 times in one animal providing an interval of 5 minutes between each application, and an average of 3 latency readings was taken as a measure of threshold to heat stimulus in area A (Fig. 1). The procedure was repeated for scrotal skin (Fig. 1, B) and the ventral tail root (Fig. 1, C) allowing an interval of 15 minutes between stimulus application to each area. Special care was taken to distinguish between casual movements from escape in response to heat stimulus (nocifensive behaviour). In the preliminary experiments, the intensity of radiant heat was set at 20% and the latency readings were very variable. The intensity was later adjusted to 40% to obtain more consistent latency readings. After the test, animals were returned to their respective cages.

Measurement of threshold to mechanical stimulus (to assess mechanical allodynia): After 1 hour of resting time, the animals used for heat stimuli experiments were tested to assess the threshold to mechanical stimulus. Rats were placed in Plexiglas[®] cubicles on a wire mesh platform (IITC Life Sciences, CA) for 30 minutes to acclimatise. A se-

ries of von Frey filaments (North Coast Medical Inc., CA) with increasing bending forces (range: 0.006 g to 10 g) were applied one by one to the skin between the penis and the scrotum (Fig. 1, A) until the animal responded to one of the filaments by moving from the original position. The bending force of the filament to which the animal responded was taken as the baseline threshold to mechanical stimulus. Similarly the threshold to mechanical stimulus was determined in the scrotal skin overlying the testicle (Fig. 1, B) and the ventral tail root (Fig. 1, C), allowing 15 minutes interval between the testings in each area. Special care was taken to distinguish nocifensive movement of the animals from casual movements.

After measuring the pain behaviour, animals were returned to their respective cages. The testings were repeated at 72 h and 1 wk.

v) Validation of the Model

From the behavioural data, 48 h time point that showed maximal reduction in heat threshold (thermal hyperalgesia) or 72 h time point that showed maximal reduction in mechanical threshold (mechanical allodynia) were chosen to carry out the validation of the model. Baseline heat thresholds were determined in two 'heat' groups of rats (heat morphine group $n = 4$; heat control group, $n = 4$), and baseline mechanical thresholds were determined in two separate 'mechanical' groups of rats (mechanical morphine group $n = 4$; mechanical control group, $n = 4$), as explained above. All animals were then injected with carrageenan in the prostate as described previously and allowed to recover. Heat thresholds were measured at 48 h in the 'heat' group to verify the presence of heat hyperalgesia, and mechanical thresholds were measured in the 'mechanical' group at 72 h to verify the presence of mechanical allodynia. The treatment groups (heat and mechanical) were injected with 5 mg/kg of morphine intraperitoneally (i. p.) and the control groups received normal saline. Heat and mechanical thresholds were measured again in the respective groups of animals 30 min after morphine treatment to see if the pain behaviour was reduced. The dose of morphine used for validation was chosen on the basis of previous studies (Rodrigues-Filho et al., 2004; Skyba et al., 2005).

vi) Statistical analysis

Multivariate ANOVA followed by Tukey's test was used to compare heat and mechanical thresholds in inflamed vs. sham animals at different time points (Before and 48 h, 72 h and 1 wk). One-way ANOVA followed by Tukey's posthoc test was used to analyse the effects of morphine on heat and mechanical thresholds.

Results and Discussion

Animals injected with 3% carrageenan showed a statistically significant reduction in mechanical threshold in the scrotal skin (site B, Fig. 1) at 72 h and 1 wk time points compared to the sham animals (Fig. 2). There was no statistically significant reduction in heat threshold in the inflamed animals when radiant heat with 20% intensity was used in the preliminary experiments (data not shown). The individual readings were highly inconsistent with 20% intensity of radiant heat. However testing at a higher intensity (40%) showed a significant reduction in heat threshold at 48 h and 1 wk time points in inflamed animals compared to sham (Fig. 3). Testing on site A and C (Fig. 1) did not show consistent reductions in heat or mechanical thresholds. Reductions in thresholds to mechanical or heat stimuli in the pelvic areas after inflammation are interpreted as heat hyperalgesia and mechanical allodynia respectively for the purpose of this study.

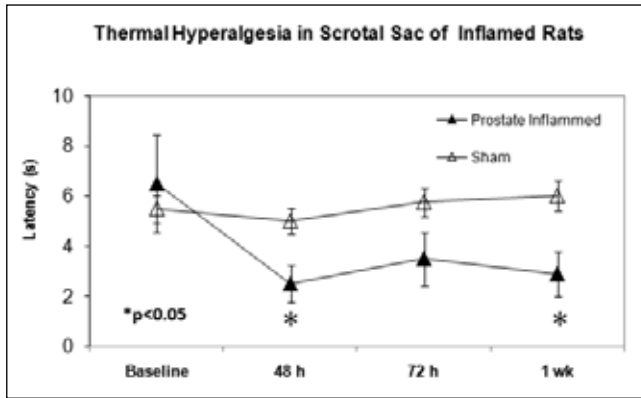


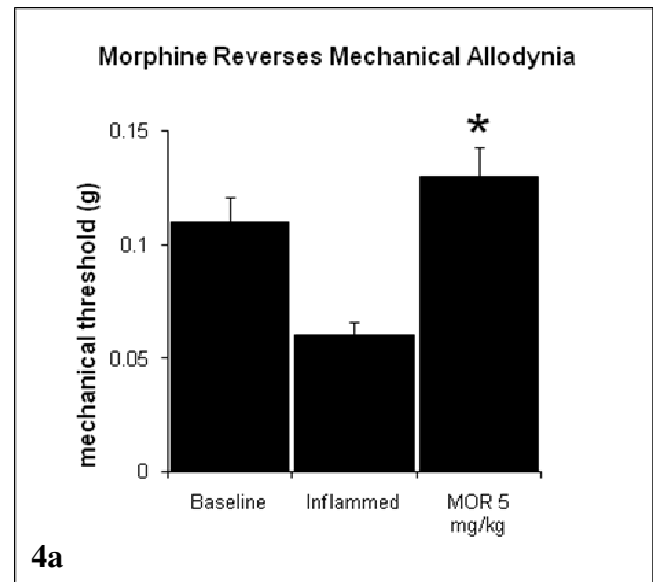
Fig. 3. Injection of 3% carrageenan into the rat prostate significantly reduced the threshold to heat stimulus applied to the scrotal skin, at 48h and 1 wk after the injection. The reduction did not reach statistical significance at 72h time point. *significantly different from non-inflamed sham animals ($p < 0.05$).

Morphine (5 mg/kg, i.p.) significantly increased the threshold to heat and mechanical stimuli in the scrotal sac skin of prostate-inflamed animals (reversed thermal hyperalgesia and mechanical allodynia) in the respective groups of animals (Fig. 4). Morphine did not produce any visible CNS inhibitory effects or catalepsy at this dose. Normal saline did not have any effects on thermal hyperalgesia or mechanical allodynia (data not shown).

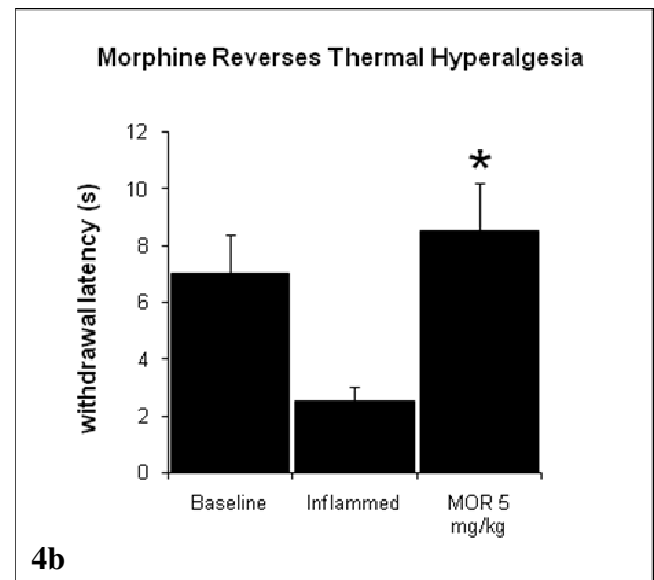
In the current study we developed an animal model of pelvic pain due to prostate inflammation. The pain behaviour was measurable objectively in this model using two different methods. Also, the pain behaviour was sensitive to morphine, a standard analgesic agent, which confirms that the nocifensive behaviour observed was indeed indicative of pain.

In the current study, right and left ventral lobes were inflamed due to the ease of surgical access. Direct measurement of pain in the prostate gland is not possible in freely moving animals. Therefore thresholds to heat stimulus (radiant heat) and mechanical stimulus (von Frey filaments) were measured in areas relevant to human pelvic pain such as area between the penis and scrotum (Fig. 1, A), scrotal skin covering the testicle (Fig. 1, B), and the ventral tail region close to the scrotum and perineal area (Fig. 1, C), before and at various time points after inflammation. Reductions in thresholds to heat or mechanical stimuli in the pelvic areas after inflammation are interpreted as heat hyperalgesia and mechanical allodynia respectively for the purpose of this study. Also, since the inflammation is induced in the prostate and the response to heat or mechanical stimuli was measured from the skin in the pelvic area, the measured parameter should be considered as secondary hyperalgesia, which is taken as a measure of primary hyperalgesia or the pain in the inflamed prostate. Occurrence of secondary hyperalgesia or allodynia in 'referred' areas of the inflamed tissue or organ has been previously reported in animals and humans (Zhang et al., 2002; Radhakrishnan et al., 2003; Vera-Portocarrero et al., 2003; Stawowy et al., 2004; Rodrigues et al., 2005; Shin et al., 2006).

In humans, the pain in the prostate is usually 'referred' to the lower back, testicles and pelvic areas (NIH-CPSI). Further, in SD rats injected with capsaicin in the prostate, neurogenic inflammation was observed in the scrotum and



4a



4b

Fig. 4. A. Intraperitoneal injection of morphine (5 mg/kg) reversed the reduction in mechanical threshold (mechanical allodynia) observed in inflamed animals. B. Intraperitoneal injection of morphine (5 mg/kg) reversed the reduction in heat threshold (thermal hyperalgesia) observed in inflamed animals. *significantly different from un-treated inflamed animals ($p < 0.05$).

tail root, indicated by extravasation of Evan's blue in inflamed animals (Ishigooka et al., 2000). It was also shown that capsaicin injection into the prostate causes c-fos expression in L6 to S1 segments of the spinal cord where the primary nociceptive (pain sensing) afferent neurons from the prostate, pelvic area, scrotum tail root converge (Ishigooka et al., 2000). In other words, the dermatomes for L6 to S1 spinal segments, where nociceptive afferents from prostate mainly converge, include the pelvic region and the tail root. Hence, heat hyperalgesia and/or mechanical allodynia were expected to occur in the pelvic area, scrotum and tail root in this model.

Technically it is difficult to apply the stimuli to the perineum of a rat due to its anatomical position and due to the construction of the testing device. Therefore the ventral side of the tail closer to the scrotum (tail root) was chosen instead. Pelvic pain due to prostatitis is often chronic and hence it was important to assess the chronicity of the proposed pain model. For this purpose, pain behaviour was assessed periodically up to 1 week. We are hoping to test for pain behaviour up to 8 weeks in future studies to further establish the chronicity of this model.

Since the pain behavior in each animal were tested at various time points (48h, 72h, 1 wk) in this study, a logical concern will be that the application of repeated noxious stimuli might have caused sensitisation of the skin to these stimuli. However, this was not the case in this model, as we did not observe any reductions in thresholds in sham animals which underwent similar testing procedures as the inflamed animals. Also, it has been shown in previous studies that this method of testing does not cause sensitisation, learning, or conditioning in animals to repeated stimuli (thermal or mechanical). The secondary hyperalgesia produced after inflammation of tissues, or injury to neurons, has been shown to sustain for longer periods (up to 7 weeks or 56 days) with repeated testing (Radhakrishnan et al., 2003; Dowdall et al., 2005).

One of the problems in this study was the absence of consistent reductions in pain thresholds in two (Fig 1. A and C) of the three pelvic areas selected for testing. In some animals, we indeed observed reduced thresholds to heat and mechanical stimuli in these areas. In some animals it was difficult to access the areas due to the positioning of the anatomical area when the animal lies down. Another reason for the variance could be the difference in the thickness or nature of scaly skin in the ventral tail root of the different rats tested. However in the scrotal sac of inflamed animals, which is one of the areas where pain is felt in human conditions of prostate inflammation, we were able to observe consistent reductions in pain thresholds, as explained in the results section.

Validation of a model is crucial while characterising any preclinical disease model. In the current study it was important to show that the escape behaviour (nocifensive behaviours) of the animals in response to radiant heat or mechanical stimulus was indeed due to pain and not due to other sensations like for e.g. tickling. Therefore the pain behaviour which was seen in this model, was validated using morphine, a standard analgesic drug, which is commonly used for validating pain models (Coderre et al., 1993; Giamberardino et al., 1995; Vera-Portocarrero et al., 2003; Whiteside et al., 2004). Morphine significantly reduced both heat hyperalgesia and mechanical allodynia in this model. Thus we showed that the nocifensive behaviours observed in this study were reliable indicators of secondary pain, and that the model may be used to screen novel drugs intended for treating pelvic pain caused by prostate inflammation.

Conclusion

In the current study, we report the development of a novel animal model of pelvic pain due to prostate inflammation, which was validated using morphine. We believe that this

model will serve as a useful tool to study the basic mechanisms underlying the development and maintenance of pelvic pain due to prostate inflammation (NIH Category IIIA).

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