Effective Concentration of Dipyrone in Drinking Water in a Rat Model of Post-Operative Pain

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ABSTRACT

Effective reduction of post-operative pain influences surgical outcome and recovery. We determined the effective analgesic concentration of dipyrone in drinking water in a post-operative pain model. Forty male Sprague-Dawley rats underwent incision and suturing of the plantaris muscle of the hindlimb paw under anesthesia. The rats were randomized to receive one of four treatments: subcutaneous saline (Group 1/ control); intraperitoneal injection of morphine 5 mg/kg (Group 2/positive control) administered on days 0, 1, and 2 post-operatively; subcutaneous buprenorphine 0.3 mg/kg immediately **pre-operatively** and dipyrone 10 mg/ml and 20 mg/ml, in drinking water, immediately after the VF measurement of study day 0, and on study days 1 and 2 (Group 3 and Group 4, respectively).

. Pain response was evaluated by mechanical allodynia 24 hours before surgery (baseline), and 3, 24, and 48 hours post-operatively. No statistically significant reduction in mechanical pain sensitivity was observed in saline-treated rats (control) or in animals treated with dipyrone 10 mg/ml on post-operative days 1 and 2 ($p>0.05$), whereas animals treated with dipyrone 20 mg/ml showed statistically significantly reduced pain sensitivity compared to the saline-treated group on both days ($p<0.05$). Treatment with morphine 5 mg/kg showed a statistically significant increase in the mean withdrawal force threshold compared to saline on all post-operative testing days ($p<0.0001$). Buprenorphine administered immediately before the surgery showed a statistically significant increase in the mean withdrawal force threshold in Group 3 vs. the saline-treated group ($p<0.05$). In conclusion, treatment with oral 20 mg/ml dipyrone post-operatively was effective in significantly reducing the pain sensitivity of laboratory rats.

Keywords: Dipyrone; Analgesia; Post-Operative Pain; Surgery.

INTRODUCTION

The accurate identification and assessment of pain in research animals undergoing painful procedures is essential for refining their care (1, 2). Animal ethics committees and regulatory authorities require researchers and laboratory animal veterinarians to assess and manage pain in animal subjects (2-4). Therefore, the management of pain in laboratory animals is an ethical obligation whose underlying principle is that causing pain and distress to animals is permissible but requires strong justification (5).

In addition to ethical issues, adequate control of surgical pain is extremely important, because, it significantly influences surgical outcome and recovery (6). Identification and prevention of pain are not straightforward issues, especially in relationship to research rodents, because they are prey species. As such, their response to pain and stress is different than that of predator species or domesticated animals

(7). For example, they often actively hide pain behavior, especially if they sense a predator (7-9). After a surgical procedure, rodents must be given analgesics for several days to achieve adequate pain relief. However, rodents are often given insufficient analgesia due to a lack of pain indicators (10). For this reason, stimulus-evoked (von Frey [VF] test, Randall-Selitto test, Hargreaves test) and non-stimulus evoked behavioral methods (grimace scales, nesting, burrowing, weight bearing, gait analysis) have been developed to quantify nociception and pain-like behaviors in laboratory animals (11).

Dipyrone (also known as metamizole and novaminsulfone), is an anti-inflammatory, antipyretic and analgesic pyrazolone derivative from a group of nonopioid nonacidic analgesics. Dipyrone is prohibited for human use in some countries due to the rare risk of reversible but potentially fatal agranulocytosis (12).

This drug is also approved for veterinary use in several countries, including in the EU, the USA and Canada, though some countries have prohibited its use in food-producing animals due to safety concerns for humans.

Analgesia is mostly administered to rodents, subcutaneously, or by an intraperitoneal injection (13, 14); however, as injections and handling may cause distress in rodents (15-19), which in turn may affect experimental parameters, it might be preferable to administer drugs by voluntary oral intake (20).

Dipyrone is soluble in water and hence it is suitable for oral administration (21). In addition, its mechanism of action is different to that of opioids and non-steroidal anti-inflammatory drugs (NSAIDs), it can be administered as part of a multimodal analgesic regimen.

In this study we investigated whether administration of oral dipyrone had a similar analgesic effect as subcutaneous administration of morphine in relieving post-surgical pain in rats.

MATERIALS AND METHODS

The study was carried out at the facilities of MD Biosciences Innovalora Ltd. (Rehovot, Israel), following the approval of the National Committee for Ethical Conduct for the Care and Use of Laboratory Animals (approval number MD-1-1077-4121).

Animals and housing conditions

Forty male Sprague Dawley rats (Envigo RMS [Israel] Ltd., Jerusalem, Israel) weighing 180-200g were randomly assigned to cages on the day of arrival and acclimated for 5 days. During acclimation period and for the duration of the study, the animals were housed in polypropylene cages within a limited access rodent facility and kept 2 rats per cage. The cages were fitted with solid bottoms and filled with sterile wood shavings as bedding material (Teklad Laboratory Grade Sani-Chips, Envigo Bioproducts Inc. Madison, WI, USA). Automatically controlled environmental conditions were set to maintain temperature at 17-23°C with a relative humidity of 30-70%, a 12:12 hour light:dark cycle and 15-30 air changes per hour in the study room. A commercial, sterile rodent diet was provided *ad libitum* and drinking water was supplied to each cage via polyethylene bottles with stainless steel sipper tubes.

Surgical procedure

The animals were anesthetized with intraperitoneal ketamine 90 mg/kg (Bremer Pharma GmbH, Warburg, Germany) and xylazine 10 mg/kg solution (Eurovet Animal Health B.V., Bladel, the Netherlands). A 1-cm longitudinal incision over the plantar surface of the left hind paw was performed, and the plantaris muscle was incised longitudinally. Following surgery, the incision was closed with two interrupted silk 3-0 stitches (DemeTech, Miami, FL, USA), and the rats were then allowed to recover from general anesthesia for about 1 hour.

Randomization to treatment groups and administration of analgesia

At baseline, prior to the surgery, ten animals were assigned to one of four experimental groups. The animals were assigned such that each treatment group had similar baseline mean body weight and mechanical allodynia test results. The number of animals per group was the minimum number required to achieve meaningful results in evaluating the effect of analgesia (22).

Each dosing group was kept in separate cages to avoid cross-contamination which could occur through consumption of fecal matter. Each animal was weighed on the day of the operation before any procedures were carried out.

Group 1 (control) received 0.2 mL saline (Medi

Table 1. Experimental groups and administered analgesia

Group Number	Group Size	Treatment	Route	Dose	Dose Volume	Dosing Regimen	Test
1	N=10	Saline 0.9%	SC	NA	NA	Once on study day 0	Von Frey test on study day -1 (baseline), and days 0 (3 hours post dosing), 1 and 2
2	N=10	Morphine	IP	5 mg/kg	5 ml/kg	On testing days; 1 hour prior to Von Frey test	
3	N=10	Buprenorphine	SC	0.3 mg/kg	5 ml/kg	Immediately before the surgery on day 0	
		Dipyrone	In drinking water	10 mg/ml	NA	3 hours post-surgery	
4	N=10	Buprenorphine	SC	0.3 mg/kg	5 ml/kg	Immediately before surgery on day 0	
		Dipyrone	In drinking water	20 mg/ml	NA	3 hours post-surgery	

IP = interperitoneally; SC = subcutaneously; NA = not applicable.

Market, Emek Hefer, Israel) subcutaneously immediately after the operation. Group 2, which served as a positive control, received an intraperitoneal injection of **morphine 5 mg/kg** (HBM Pharma s.r.o., Martin, Slovakia) post-operatively – one hour before the VF test on testing days 0, 1 and 2. Group 3 and 4 received buprenorphine 0.3 mg/kg (Richter Pharma, Wels, Oberosterreich, Austria) immediately after the surgery and dipyrone (Vetoquinol, Lure, France) 10 mg/ml (group 3) and 20 mg/ml (group 4), respectively, was added to their drinking water 3 hours after the operation (Table 1).

Assessment of pain

Pain response was evaluated by mechanical allodynia test using the Von Frey (VF) apparatus (Touch Test Sensory Evaluator, Kit of 20, Stoelting Co. Wood Dale, IL, US) (11). Allodynia is characterized by a heightened sensitivity to stimuli that are not typically painful. The animals were accustomed to the VF apparatus for two days before the surgical procedure. The VF test was performed 24 hours before the operation (baseline), and 3, 24 and 48 hours post-operatively.

For the VF test, the rat was placed in an enclosure and positioned on a metal mesh surface but allowed to move freely. The rats' cabins were covered with red cellophane to diminish environmental disturbances. The test began after cessation of exploratory behavior. A monofilament was applied perpendicularly to the plantar surface of the hind paw for 2–5 seconds until it buckled. The set of VF monofilaments provide an approximate logarithmic scale of actual force and a linear scale of perceived intensity. A response was considered positive if the animal pulled back its paw during application of the stimulus. A decrease in the withdrawal force threshold reflects heightened sensitivity, indicative of allodynia, meaning that even a gentle stimulus is enough to provoke a reaction (23).

At the end of the experiment (2 days after surgery), the rats were humanly euthanized with pentobarbital sodium (Chanelle Pharma, Loughrea, Ireland).

Statistical analysis

Statistical analysis was performed using GraphPad version 8.0 (Boston, MA, USA). The data were analyzed using descriptive statistics. Mechanical allodynia was analyzed by mean and standard error of the mean (SEM) and compared among groups using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. A p value less than 5% was considered statistically significant.

RESULTS

The mean body weight of the rats was similar among the treatment groups (Table 2). One rat from Group 2 died one day after the operation due to a technical cause.

The results of the mechanical allodynia test are shown in Table 3. Animals treated only with saline post-operatively did not show any significant reduction in the mechanical pain sensitivity (i.e., increased withdrawal force threshold). In contrast, treatment with morphine 5 mg/kg resulted in a statistically significant increase compared to the saline treated group in the mean withdrawal force threshold on

Table 2. Mean weight (g) of the animals at baseline by treatment group

Group number	N	Treatment Group	Mean ± SEM
1	10	Saline 0.9%	240.60 ± 2.52
2	10	Morphine 5 mg/kg	238.00 ± 3.57
3	10	Buprenorphine 0.3 mg/kg + Dipyrone 10 mg/ml	237.80 ± 2.37
4	10	Buprenorphine 0.3 mg/kg + Dipyrone 20 mg/ml	243.40 ± 2.16

SEM, standard error of the mean

Table 3. Comparison of pain response by mechanical allodynia using the Von Frey apparatus (Touch Test).

Group number	Treatment group	Baseline	Day 0	Day 1	Day 2
		Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
1	Saline 0.9% (N=10)	26.00 ± 0.00	4.60 ± 0.43	5.00 ± 0.54	4.80 ± 0.44
2	Morphine 5 mg/kg (N=10)	26.00 ± 0.00	23.56 ± 1.62**,†	18.78 ± 2.37**,†	17.67 ± 2.69**,†
3	Buprenorphine 0.3 mg/kg+ Dipyrone 10 mg/ml (N=10)	26.00 ± 0.00	11.00 ± 2.52*	6.40 ± 0.58	7.20 ± 0.61
4	Buprenorphine 0.3 mg/kg + Dipyrone 20 mg/ml (N=10)	26.00 ± 0.00	7.40 ± 0.73	9.90 ± 0.71*	11.0 ± 1.72*

* $p<0.05$ vs. control group (saline 0.9) using one-way ANOVA followed by Dunnett's post-hoc test.
** $p<0.0001$ vs. control group (saline 0.9%) using one-way ANOVA followed by Dunnett's post-hoc test.
† N=9
SEM, standard error of the mean

all post-operative testing days 0, 1 and 2 ($p<0.0001$ for the comparison between the group treated with morphine and the group treated with saline on each post-operative day, Table 3).

Treatment with buprenorphine immediately after the operation (Groups 3 and 4), resulted in a statistically significant increase in the mean withdrawal force threshold only in group 3 when compared to the saline treated group (11±2.52g vs. 4.60±0.43, $p<0.05$).

Animals treated post-operatively with dipyrone 10 mg/ml in drinking water did not show any statistically significant reduction in mechanical pain sensitivity post-operatively on days 1 and 2, whereas treatment with 20 mg/ml dipyrone in the drinking water 1 and 2 days post-operatively resulted in a statistically significant increase in the mean withdrawal force threshold compared to the saline-treated group (9.90±0.71g vs. 5.00±0.54g on post-operative day 1 and 11.00±1.72g vs. 4.80±0.44g on post-operative day 2; $p<0.05$ for the comparisons on both days).

DISCUSSION

The use of analgesia is critical for preventing or limiting pain in experimental procedures and to guarantee the welfare of research animals, however, there is a lack of information on pain management in laboratory animals. We investigated whether administration of oral dipyrone has the same analgesic effect as intraperitoneal administration of morphine in relieving post-surgical pain in rats using the VF test.

The dipyrone doses were chosen according to prior reports in the literature (24). We did not exceed 200 mg/kg (20 mg/ml) in the drinking water due to the bitterness of the dipyrone oral solution, which would have prevented the rats from drinking the water in which it was mixed. We did not add sugar to the drinking water to encourage drinking, to prevent the rats from gaining weight, as this would be undesirable in certain types of experiments, such as those investigating metabolic conditions. For the same reason, we did not add analgesics to flavorful food, so as not to encourage excessive eating and obesity.

Our results demonstrated that treatment with dipyrone 20 mg/ml in drinking water 1 and 2 days after surgery, reduced the pain sensitivity of treated rats. While morphine showed the greatest reduction in pain sensitivity post-operatively, it also induces sedation in high doses which may interfere with post-operative evaluations of laboratory rodents (e.g., assessment of certain behaviors). Ince *et al*. have shown,

that administration of oral dipyrone 250 mg/kg or 500 mg/kg to male albino Wistar rats was more effective than oral paracetamol 250 mg/kg and 500 mg/kg for treating surgical trauma-related pain, inflammation, and oxidative stress; however, they did not examine the effect of this relatively high dipyrone dose on the animals' behavior (25). In a study that evaluated dipyrone on behavioral parameters of naïve male Wistar-Han rats, administration of subcutaneous dipyrone 177.8 mg/kg three times/day for 4 days diminished exploratory behavior, but did not remarkably affect grooming, suggesting that dipyrone has a suppressive motor effect (26).

To achieve balanced analgesia, different substance classes should be combined to produce the best possible analgesia with the fewest possible effects on behavior and other side effects. Co-administration of subcutaneous dipyrone 177.8 mg/kg and tramadol 17.8 mg/kg three times a day for 4 days resulted in nociception but a trend for tolerance was observed only after the second injection (26). Alemán-Laporte *et al.* (27) have evaluated the effects of intraperitoneal injections of dipyrone 178 mg/kg, tramadol 17.8 mg/mL, and meloxicam 1.5 mg/kg – alone and in combination – on the behavioral parameters of naïve male Wistar-Han rats. They showed that administration of dipyrone and tramadol caused a generalized inhibition of behavior in the treated rats by reducing locomotion, rearing and all subtypes of grooming and have suggested that there is synergism between these two drugs that affects rat behavior in comparison to the effect of dipyrone alone. In contrast, in the same study, rats treated with tramadol or with combined tramadol and meloxicam showed exploration behavior that was similar to or higher than that of rats that were treated only with saline (27). In a meta-analysis that examined the effect of post-surgical administration of a single analgesic or analgetic drug combinations on mouse and rat behavior, the most frequently used drugs in rat studies included NSAIDs (carprofen, (dex)ibuprofen, ketoprofen, meloxicam) and opioids (buprenorphine, nalbuphine, morphine, fentanyl, tramadol), and these were usually administered intraperitoneally or subcutaneously. Most studies have evaluated the effect of post-surgical analgesia on grimace scales, while the effects on nest building and burrowing were less studied. The authors concluded that there are no universally valid analgetic concepts applicable to all experimental interventions (28).

Notably, it has been reported that the animal's strain, age and sex may also affect its reaction to pain in specific pain evaluation tests as well as its integrated behaviors (28-31). Jourdan *et al.* have shown increased mechanical sensitivity to pain and no change in thermal sensitivity for old (26 months) Lou/c rats compared to mature (4 months) and middle-aged (18 months) rats. They also showed that the effect of morphine significantly decreased with age while paracetamol, aspirin or clomipramine had no age-related effect (32).

In this current study we chose the VF test as it allows to measure pain directly and is not affected by other factors such as blood pressure, heart rate or pulse. The Randall-Selitto assay is another technique to assess aversive behaviors in responses to mechanical stimuli by uniformly increasing pressure on the paw. The intensity of pressure causes an escape reaction defined as the withdrawal threshold. However, the mechanical stimulation differs from that of the VF test, which may also activate low-threshold mechanoreceptors in addition to nociceptors (11). However, these stimulus evoked methods may cause additional stress to the animals resulting in concerns regarding their translational applicability to the clinical field (11).

This study has several limitations: Firstly, animal groups were limited to 10 animals per group consistent with the reduction principle. Secondly, after the surgery the rats in each treatment group were housed together and we did not measure the amount of water consumed by the animals, nor did we compare the amount of water consumed by each treatment group. This will have affected the dose of dipyrone consumed in the treatment groups treated with this analgesic. Although sweetening of non-palatable drugs is often necessary to facilitate their consumption by rodents, Tang *et al* (33). have found that mice did not consume significantly less water when it was supplemented with dipyrone. Nevertheless, reduced intake of food and water are known side effects of several analgesics including buprenorphine and may also suggest post-surgical pain (34, 35). Administering dipyrone by gavage could have helped to better determine its effective analgesic concentration. In our study, buprenorphine was administered Immediately before surgery. Although it should have reduced the immediate post-surgical pain, the rats may have moved less – either due to its effect or due to post-surgical pain – and therefore may have consumed less food and water, and subsequently – received a lower dipyrone dose by water than planned. The concentration of the analgesics or their metabolites in the animal's blood were not measured to prevent additional stress to the animals. Therefore, the

exact dose of dipyrone providing analgesia could not be determined. Third, we did not use readout scores to measure distress scores, grimace scores, burrowing, or nesting activity, which have been used to evaluate post-surgical pain in mice and rats (28, 36-38). Tang *et al*. did not find significant differences in the distress score, burrowing, or nesting activity when administering dipyrone to mice (33). Fourth, only male rats were included in the study. Studies have shown that female rats tend to have lower pain thresholds, and are less responsive to morphine (39-41), and buprenorphine (42) administration. Fifth, we could not distinguish between the animal's pain and other causes of stress: the tests were performed during the daytime, which may have affected the rat's responsiveness and stress levels. Furthermore, the handling of the animals during the VF test may have caused additional stress. Sixth, as the analgesics administered have different mechanisms of action, their effects on the animals may have affected their response to the VF test. Last, our study only evaluated the short-term effect of pain reduction following surgery as the animals were sacrificed two days after surgery.

In conclusion, under the conditions of this study, and in agreement with the in-life data, treatment with dipyrone 20 mg/ml in drinking water post-operatively reduced the pain sensitivity of male laboratory rats. Since its mechanism of action differs from that of opioids and non-steroidal anti-inflammatory drugs, it can be included as part of a multimodal analgesic regimen for post-operative pain management. Further studies are required to determine the exact dose of dipyrone that provides analgesia in this model.

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