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ANALGESIC POTENTIAL OF BUPRENORPHINE IN AN EXPERIMENTAL BONE DEFECT MODEL ANALYSED THROUGH REAL TIME RGS

POTENȚIALUL ANALGEZIC AL BUPRENORFINEI ÎNTR-UN MODEL EXPERIMENTAL DE DEFECT OSOS ANALIZAT PRIN RGS ÎN TIMP REAL

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ABSTRACT | REZUMAT

In Romania, pain assessment, in laboratory animals, in invasive orthopaedic surgery is still considered a setback. For rats, buprenorphine, in addition to other opioids is suggested for alleviation of moderate to severe pain, but few relevant supporting data is available. This paper investigates the analgesic potential of buprenorphine in an experimental bone defect in rats. 12, male, Wistar rats were used, 9 were subjected to a non-critical surgically induced bone defect while three were left as reference group. The efficiency of two Buprenorphine doses (0.01 and 0.03 mg/kg) was determined using intraoperative pain assessments (skin incision, muscular dilacerations, defect drilling, layers suture), as well as postsurgical assessment. Postsurgical evaluation was done using a real time RGS scoring method for 8 hours. Control animals presented intraoperative sensibility during muscular dilacerations and defect drilling. In the postoperative period the bone surgery produced moderate pain (0.5-0.9). In the analgesia groups, both buprenorphine doses successfully reduced pain (0.09-0.3) starting at 2 hours up to 8 hours postoperatively. Despite both doses having analgesic potential compared to the control group, a more optimal effect is conferred by the dose of 0.03 mg/kg (0.09-0.2) throughout the whole experimental period. These results offer unprecedented evidence for the need of analgesia during surgically induced bone defects in rats, highlighting the usefulness of the RGS method for the assessment of animal suffering in surgical experimental procedures.

Keywords: buprenophine, bone defect, pain scoring, RGS

Lipsa datelor cu privire la intensitatea durerii animalelor de laborator în cazul unor intervenții chirurgicale ortopedice invazive este o deficiență majoră din punct de vedere al conceptului de 3R în România. Buprenorfina, în comparație cu alte opioide, este recomandată la șobolani pentru ameliorarea durerii moderate, cât și a celei severe, însă există puține date relevante care să sprijine aceste afirmații. Prezenta lucrare are ca scop investigarea potențialului analgezic al Buprenorfinei în cazul unui defect osos indus experimental, la șobolani. S-au utilizat 12 șobolani, masculi, Wistar, din care 9 au fost supuși unui defect osos non-critic, în timp ce alți 3 șobolani au alcătuit grupul de referință. S-a determinat eficacitatea a două doze diferite de Buprenorfină (0,01 și 0,03mg/kg) în comparație cu grupul control operat, dar fară terapie antialgică, evaluându-se durerea la fiecare nivel al actului operator (incizia pielii, dilacerarea musculaturii, forajul defectului osos și suturarea pe straturi), dar și postoperator. Evaluarea postoperatorie a fost făcută folosind metoda trăsăturilor faciale (RGS), timp de opt ore. Animalele din grupul de control au prezentat sensibilitate intraoperatorie în timpul dilacerării musculaturii și în momentul efectuării defectului osos. În perioada postoperatorie chirurgia osoasă a produs durere moderată (0,5-0,9). În cazul grupurilor care au primit analgezie, ambele doze de Buprenorfină au redus cu succes durerea (0,09-0,3) începând cu 2 până la 8 ore postoperator. În ciuda dozelor cu potențial analgezic comparativ cu grupul de control, efectul optim scontat este dat de doza de 0,03 mg/ kg (0,09-0,2) pe întreaga perioadă experimentală. Aceste rezultate oferă dovezi fără precedent pentru necesitatea analgeziei în timpul defectelor osoase induse chirurgical la șobolani, subliniind utilitatea metodei RGS pentru evaluarea suferinței animalelor în procedurile experimentale.

Cuvinte cheie: buprenorfină, defect osos, scor de durere, RGS

Pain assessment is a critical part of animal welfare. It is common knowledge that the animal welfare paradigm states that the animals should be healthy, well fed and housed, free of stress, fear and pain (7).

This extends to animal-based research through the explicit set of principles that guide the ethical evaluation of animal use, the 3Rs principles (Replacement, Refinement and Reduction). Refinement accounts for improving laboratory animal welfare, which is vital for

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normal biological functioning and behavioural repertoire (15). Therefore, a challenging area in the field is refining procedures which could reduce the degree of stress and pain in different experimental models. The Rodent grimace scale (GS) was recently validated for pain assessment in rats and mice; therefore, our ability to detect pain and unethical conditions in translational research has increased (1, 3, 5). Although Rat Grimace Scale (RGS) has been widely applied to observe and diagnose acute pain, no study to our knowledge has yet investigated the pain derived from a rodent non-critical bone defect. This particular area of biomaterial testing in bone regeneration has been neglected from the refinement concept point of view. *In vivo* bone defects are essential tools for certifying the biocompatibility, biophysical effects and biosafety of biomaterials in regenerative medicine (6). However, surgery, anaesthesia and tissue damage cause a physiological stress response and pain. The treatment of pain itself varies and could lead to behavioural changes (19). Assessing the effectiveness of certain analgesic drugs in specific experimental models (tissue engineering models, metabolic models, and cancer models) has been limited. Buprenorphine is known to be a potent analgesic used in moderate to severe pain in rats (17, 14), but little data is available on perioperative pain assessment after experimental bone defects protocols in rats. This paper investigates the pain level associated with a bone defect in rats and discusses the analgesic potential of buprenorphine in this experimental context.

MATERIALS AND METHODS

The research has been carried out on 12 healthy adult male Wistar rats. The procedures involved in the present project were conducted under the guidelines of Directive 2010/63/EU and the national law 43/2015.

The rats were housed under standard conditions: temperature 23°C, humidity 55%, and light/dark cycle 12/12. The animals were fed on standard rodent granular food and they had unrestricted access to water. The study was done under the approval of the Bioethics Comity of the University of Agricultural Sciences and Veterinary Medicine of Cluj Napoca and they were authorised by the State Veterinary Authority (Authorization No. 52/30.03.2017).

Experimental procedure

As a model of pain, a non-critical bone defect (2 mm wide and 2 mm depth) was performed on 9 rats, while three were left as an absolute reference group. The surgery was performed under general anaesthesia with isoflurane (Iso-Vet, Piramal Healthcare UK Limited) in 100% oxygen. The premedication protocol was performed with Xylazine (Xylazin Bio 2%, Bioveta, Cehia, 6 mg/kg) and Ketamine (Narkamon 100mg/ml, Bioveta, Cehia, 60 mg/kg). Following shaving and scrubbing, a lateral skin incision was made to expose the left biceps femoris. After blunt dilacerations of the muscle, the bone was exposed. At a slow rotation speed, 2 mm width defects (1 defect/ animal) were drilled into the distal part of the femoral diaphysis. The defect was filled with bone cement. The muscle and skin layers were closed with an absorbable polydioxanone suture (Surgicryl 4/0) (Fig. 1). The animals were kept under close monitoring on the entire length of the study, focusing on infection prevention, using Enrofloxacin (5 mg/kg). All Buprenorphine (Buprecare 0,3 mg /ml, Animalcare, UK) (BP) injections were prepared by a third party not involved in the experiment. All injections were performed 30 minutes preoperatory, subcutaneously. The animals were divided in 4 experimental groups (G) of 3 animals/G (n=3). G1 was considered reference group and contained unoperated animals. The operated animals were randomized into

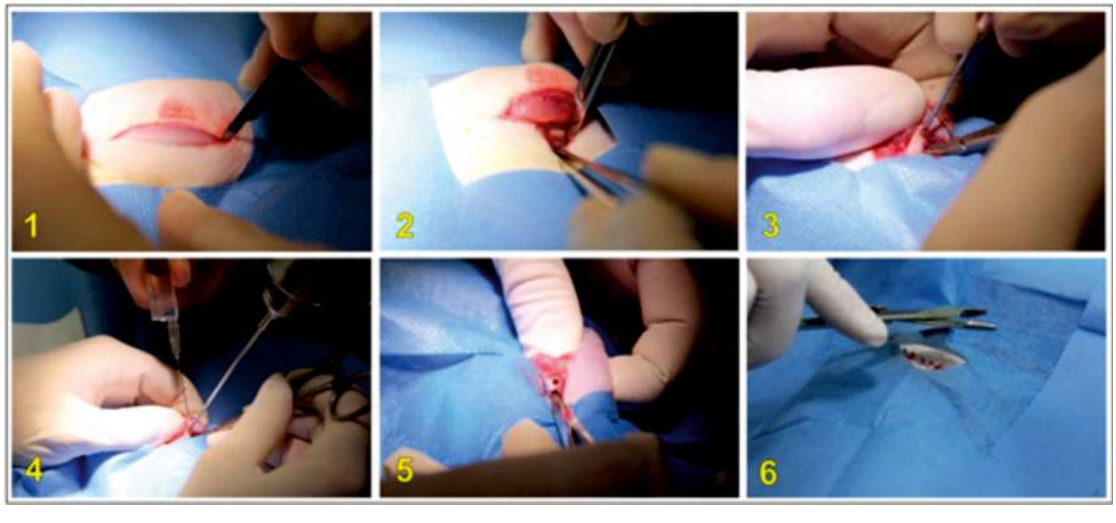

Fig. 1. Digital photograph of the surgical steps

one of the 3 treatment groups. One treatment group (G2) was considered control and received the same handling and treatment (bone defect, antibiotic, fluid) as the other groups, but the Buprenorphine was replaced by saline. The other groups (G3, G4) received either Buprenorphine (0.01 mg/kg), or Buprenorphine (0.03 mg/kg) dissolved in saline at the same volume.

Intraoperative assessment

The efficiency of the two Buprenorphine doses (0.01 and 0.03 mg/kg) was determined intraoperatively following 4 surgical steps: skin incision, muscular dilacerations, defect drilling, layers suture. Intraoperative nociception was graded as following: 0- no reaction, 1- light reaction (muscular tremor), 2- moderate reaction (localized movement), 3- intense reaction (generalized movement). Due to intraoperative reactions, the control group received rescue analgesia consisting in 10 mg/kg, i.m, of Ketamine.

Postoperative assessment

Postoperative pain intensity was measured using the Real-time RGS scoring as previously described by Leung et al, 2016 (10). Rats were placed individually in a glass cage. The facial expression of the rats was recorded by three evaluators, who were blind to the study treatment. Every evaluation was repeated every 30 seconds for a ten-minute observation period. The scores were averaged every three minutes to produce three separate scores and then these were averaged to a single one/animal/at a specific time interval.

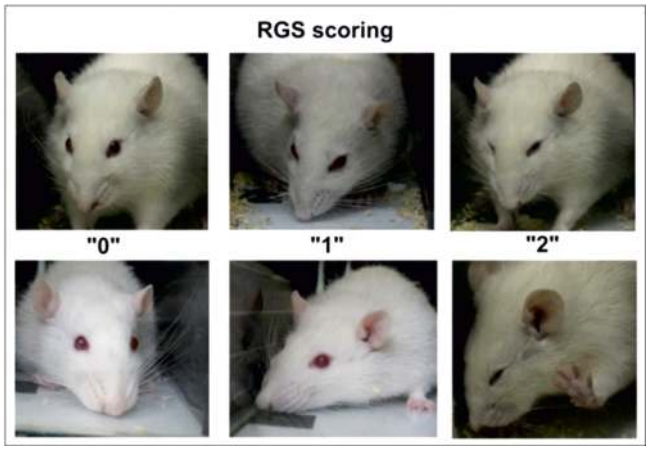

Fig. 2. Digital photographs of RGS scoring during this study

The evaluation of pain was done across at time: 0, 30 minutes, 2, 4, 6 and 8 hours post-surgery.

Real time evaluation was not performed if the rat was sleeping, rearing, sniffing or grooming. The RGS pain scale used was described by Sotocinal et al, 2011 (16) and consists of a 3-point scale: 0-no pain, 1- moderate pain, 2- intense pain, for each facial action unit.

The reference group exhibited no pain (0) (Fig. 2). After the observational time required for the current study, all the animals received daily analgesia, if needed.

Statistics

All data are reported as mean ± SD. To assume Gaussian distribution normality distribution was checked by D'Agoustino and Pearson omnibus normality test. These data did not pass the normality test, thus we used Kruskal-Wallis test, followed by post-test Dunns. Statistical significance was set at $p<0.05$ (95% confidence interval). Statistical values and figures were obtained using Graph Pad Prism version 5.0 for Windows, Graph Pad Software, San Diego California USA.

MATERIALS AND METHODS

Intraoperative analysis | The intraoperative observation highlighted that the control group (G2) presented light to moderate reactions during the surgical procedure, due to the lack of buprenorphine supplementation (Table 1). In group G3 a slight reaction was observed during muscular dilaceration expressed by muscle tremors. In both Buprenorphine treated groups (G3, G4) a slight reaction was observed during the drilling of the bone defect (Table 1).

Postoperative analysis

The most striking result to emerge from the postoperative evaluation is that the non-critical bone defect surgery determined only moderate pain level, with values ranging from 0.4 to 0.9, according to the RGS scoring system. The control group, who did not benefit from analgesic therapy revealed moderate pain during the entire observational period (Fig. 3). Buprenorphine therapy provided analgesic effect (G4-0.41) ($p<0.001$) in comparison to the control (0.76), in the dose of 0.03 mg/kg, starting with the second observational period (30 minutes post-surgery). Both Buprenorphine doses were able to diminish the pain intensity up to 9-fold during the whole post-surgery observational time, however the analgesic effect was clearly visible at 4 (G3-0.02; G4-0.09) and 6 (G3- 0.1; G4-0.09) ($p<0.001$) hours post-surgery.

DISCUSSIONS

The purpose of this study was to assess the pain level induced by a bone surgery experimental protocol and identify an analgesic treatment score for the opioid Buprenorphine, which is routinely used and recommended as analgesia for surgeries producing severe pain (18). In the past decade we have experienced a growing importance in rodent fracture/bone healing experimental models (9). The number of rodents used in *in vivo* biomaterial testing witnessed a considerable rise in the last 30 years, currently being at almost half

Table 1

Effects of Buprenorphine (BP) on intra-operative monitoring

Animals	Skin incision	Muscular dilaceration	Defect drilling	Layers sutures
G1 (no surgery)	0	0	0	0
G2	1.5±1.29	1±0.81	1.25±0.5	0.25±0.5
G3	0	0.5±0.57	0.5±0.57	0
G4	0	0	0.25±0.5*	0

(mean ± SD) (*= p>0.05 as compared to BP 0 mg/kg) (3 animals / group; used Kruskal-Wallis test, post-test Dunns) Rat Grimace Scale (RGS)

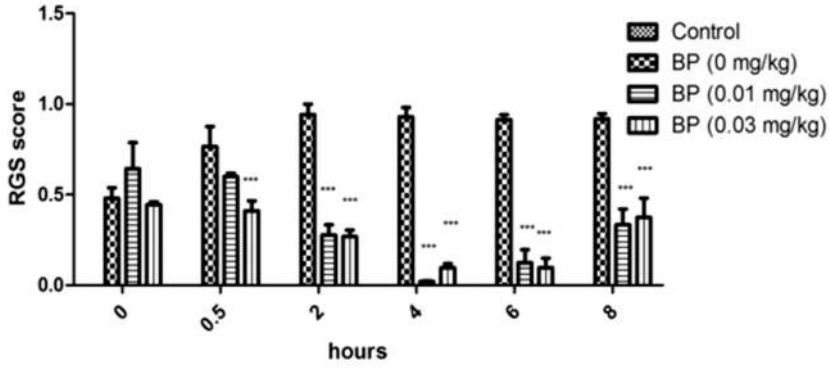

Fig. 3. Effects of Buprenorphine (BP) on post-surgery pain control (mean ± SD) (***= p>0.001 as compared to BP 0 mg/kg) (3 animals / group; used Kruskal-Wallis test, post-test Dunns) Rat Grimace Scale (RGS)

of all animal bone studies (6, 9). Few researchers have addressed the question of pain assessment in a specific experimental environment regarding bone defects and biomaterial implantation, and thus, limiting the refinement component of the 3R principle. Current solutions and guidelines are inefficient due to the lack of analgesia statements in the description of an experimental defect in research articles.

Despite knowing that surgery to repair fractures/ bone defects is classified as severe pain procedure according to the Guidelines for Evaluating and Treating Surgical Pain in Mice and Rats (13), there is still a need for an exact pain score, exact dose treatment for avoiding numerous side effects, or specialized veterinary staff. According to the concept of pre-emptive analgesia, analgesics are more effective when administered prior to surgery, because there are key surgical moments such as skin and tissue incision, where local nerves transmit impulses to the brain that are interpreted as pain (20). In our study, 4 major surgical actions were chosen, which were correlated with tissue damage. Remarkably, the use of both Buprenorphine doses managed to confer a superior intraoperative analgesia in comparison with the group which received only Xylazine and Ketamine in doses chosen by us. Rescue analgesia was necessary in order to abolish surgical pain. This substantiates previous findings in literature, where buprenorphine treatment besides general anaesthesia reduced corticosterone levels during surgery in comparison with saline treated group or local analgesic therapy (lidocaine) group (10). As reported by Roughan and Flecknell, 2002 and Franchi et al, 2007 (14, 8), the evidence we found highlights that the opioid analgesic treatment with buprenorphine enhanced sedative effects of anaesthesia and reduced stress and pain associated with surgery. Rat grimace scale is recognized as being a reliable means of assessing pain and the resultant scores are consistent over time (5, 12). RGS is used as an observational tool, but also as an analgesic interventional tool (12). Both methods described by literature, the classical method (16), but also the real time method (11) are accurate, offering refinement to the humane care of laboratory animals. Our data would seem to demonstrate that postsurgical pain induced by a non-critical bone defect is moderate. There is a strong possibility that a larger defect, a critical one, could induce severe pain. There is good agreement between our results and the guidelines provided by Committee on Pain and Distress in Laboratory Animals, in Recognition and Alleviation of Pain and Distress in Laboratory Animals, where the severity of pain regarding bones is classified as moderate to severe and the duration of pain, intermittent. Although these guidelines exist, as far as we know this is the first time an RGS score and a pain level is described in a bone experimental protocol.

Our results further widen our knowledge the fact that the efficiency of the analgesia provided by buprenorphine is dose dependent. These findings reinforce the usefulness of buprenorphine as a reliable analgesic agent in rats. Additionally, the most striking observation to emerge from the data comparison was that both buprenorphine doses (0.01 mg/kg and 0.03 mg/kg) were

able to reduce the pain intensity to almost zero at 4 and 6 hours post operatory, and also to provide proper analgesia during the whole observational period. None of the differences between the two experimental doses of Buprenorphine, in the postsurgical period, were statistically significant. Surgery induces a physiological stress response based on neural and endocrine alterations (4). It is considered that the reduction in nociception implies the reduction in stress associated with surgery, thus reducing the suffering of an animal and all consecutive alterations in its normal physiology (4). Our experiment results are in line with previous statements (10), where buprenorphine treatment reduced postoperative plasma corticosterone levels compared to local analgesics and showed an effect for up to 18 hours after surgery. The analgesic efficacy of Buprenorphine has been assessed in numerous rodent models of acute pain, but in contradiction with our findings Curtin et al, 2009 (2) stated that only Buprenorphine at 0,05 mg/kg induced postoperative isoalgesia in an acute inflammatory model of pain, whereas the dose of 0,01 mg/kg did not meet the analgesic criteria. We believe that our approach could be used in all bone experimental designs in order to rapidly evaluate pain and establish an individualized plan for pain relief. This could be useful to avoid side effects, abnormal animal behaviour and failure in experimental research.

CONCLUSIONS

This study has managed to enhance our understanding on pain in a specific experimental model in rats used worldwide in biomaterial research. This work has revealed that the level of pain induced by a non-critical bone defect in an experimental model is moderate. Both Buprenorphine doses presented are suitable for postsurgical analgesia, but only the higher dose 0.03 mg/kg offers the requirements necessary in a surgical intervention on bones. We hope our research will be helpful in ameliorating animal care and welfare during experimental procedures which involve bone structures in rats.

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