Comparative Medicine
Copyright 2019
by the American Association for Laboratory Animal Science

Vol 69, No 6
December 2019
Pages 535–545

Overview

Influence of Pain and Analgesia on Orthopedic and Wound-healing Models in Rats and Mice

Monika K Huss,[1,*] Stephen A Felt,[1] and Cholawat Pacharinsak[1]

The surgical stress response and resulting physiologic changes can lead to postoperative complications and negatively impact animal welfare. Although appropriate pain management is crucial to reduce the pain and stress response to surgery, analgesic choice can significantly affect bone and wound healing. This review aims to summarize data from rat and mouse studies and to provide recommendations for integrating analgesia into orthopedic and wound healing models in these species. Data from other species, such as humans, rabbits and other rodents, is included, where available. From these data, we conclude that for orthopedic surgical models, opioids, local anesthetics and dissociative agents have minimal impact on fracture healing; cyclooxygenase 2 (COX2) selective nonsteroidal antiinflammatory drugs (NSAID) may be used in the short-term; and steroids should be avoided. For wound healing models, short-term systemic or topical opioids have negligible impact on wound healing; NSAID or local anesthetics may be used short-term; and systemic steroids should be avoided. Alternative analgesics such as tramadol, gabapentin, ketamine, and acetaminophen warrant consideration and further evaluation for both orthopedic and wound healing models. In all cases, researchers and veterinarians should work together to determine the appropriate analgesic plan to minimize pain, as well as to minimize unwanted effects on the orthopedic and wound healing models themselves.

Abbreviations: LA, local anesthetic; cyclooxygenase (COX); PO, *per os*; ACTH, adrenocorticotropic hormone; TSH, thyroid stimulating hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone.

DOI: 10.30802/AALAS-CM-19-000013

Rats and mice as surgical orthopedic and wound healing models. Rodents, including rats (38%) and mice (15%), comprise over half of the animals used in orthopedic research.[102] Rats are commonly used as subjects for orthopedic studies of fracture, bone defect repair, bone ingrowth, bone or joint infections, osteoporosis, osteomyelitis, bone circulation, prosthetic debris, biocompatibility, and nerve repair.[6] Mice are popular as orthopedic models because of ease of genetic manipulation and are most often used to study osteogenesis, chondrogenesis, osteoporosis, arthritis, bone tumor, and nerve repair.[6] There are a number of rodent wound healing models: incisional, excisional, dead space, flap surgery, burns, aging, and dermonecrotic models in various locations including the dorsal, ventral, or neck areas, as well as the flank, leg, and feet areas.[8,39,74,79,119] Based on the assumption that what is painful to a human would be painful to another animal, analgesia should be included as part of the experimental design for all experimental surgical procedures.[20]

When using animals in research, recognition and treatment of pain are essential for both ethical and regulatory reasons. Justification must be provided to an Institutional Animal Care and Use Committee (IACUC) or similar oversight body to document both the need to perform the particular surgical procedures in live animals and the analgesic measures that will be taken to mitigate pain.[20] The US Government *Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training* states that the minimization of pain is imperative, and that procedures must be performed using "appropriate sedation, analgesia or anesthesia".[30] Likewise, the *Guide for the Care and Use of Laboratory Animals* ("The Guide") states that "The selection of appropriate analgesics and anesthetics should reflect professional veterinary judgment as to which best meets clinical and humane requirements as well as the needs of the research protocol."[62,98] In addition to being an ethical and regulatory requirement, improper pain management can also have significant implications on study outcomes and accurate evaluation of biologic effects.[66,81] However, when providing analgesia for orthopedic and wound healing models, the breadth of analgesia options cana complicate devising an analgesic plan that is effective and does not interfere with the study objectives. This challenge is further complicated by scientific literature that inconsistently reports the use of anesthetics and analgesics during experimental procedures.[21] In this review, we summarize the scientific literature available to provide recommendations for integrating analgesia into rat and mouse orthopedic and wound healing models. When only minimal data is available from rat and mouse studies, we draw from information available from other species including humans, rabbits and other rodents.

Pain and the surgical stress response. Stress has been demonstrated to elicit endogenous pain relief, a phenomenon known as stress-induced analgesia.[18] The endogenous pain inhibitory systems can be activated by fear, anxiety and stress.[2,5,104] Stress-induced analgesia depends on, intensity and duration of the stress, and the level of control the animal has over its stress.

Received: 22 Jan 2019. Revision requested: 24 Feb 2019. Accepted: 01 Jul 2019.
[1]Department of Comparative Medicine, Stanford University, Stanford, California
[*]Corresponding author. Email: monikag@stanford.edu

This suggests that the context in which the animal perceives the stressor is a major determining factor of the perceived pain.[5,58,65]

The phrase "surgical stress response" is used to describe the physiologic response (including hormonal and metabolic changes) to surgery and pain.[26,70] The 3 key components of the surgical stress response are: 1) sympathetic nervous system activation, 2) endocrine response, and 3) immunologic and hematologic changes.[25,26,33] In brief, the sympathoadrenal response results from increased catecholamine secretion. Protracted release of circulating norepinephrine and epinephrine leads to tachycardia and hypertension, affecting the function of the liver, pancreas, and kidney. The endocrine response involves changes in pituitary hormone secretion that include increased ACTH, growth hormone, and arginine vasopressin; and increased or decreased TSH, FSH and LH.[25] These changes result in secondary effects on hormone secretion in target organs, including the adrenal cortex (increased cortisol and aldosterone), pancreas (decreased insulin and increased glucagon), and thyroid (decreased thyroxine).[25] The overall outcome is increased catabolism, mobilization of energy stores, and retention of salt and water to maintain fluid volume and cardiovascular hemostasis.[25] Protein catabolism and hyperglycemia are linked to muscle wasting, weight loss, impaired wound healing, and infection.[25] The immunologic changes include increased production of cytokines and acute phase reactants, reduced recruitment of inflammatory cells to the wound margin, impaired antibacterial function and slowed healing.[28,124] A generalized state of immunosuppression following surgery or trauma is implicated in the development of septic complications postoperatively.[82,89,91] The greater the surgical stress, the more profound the effect on the immune system and healing.[82] Although the surgical stress response may represent a conserved defense mechanism,[92] the resulting physiologic changes may lead to postoperative complications and can interfere with orthopedic and wound healing.

Pain associated with surgery and the pain associated with surgical wounds is the consequence of stimulation of nociceptors and the subsequent changes in nociceptor nerve endings (C and A-δ nerve fibers) which are processed by numerous neuronal networks and brain structures.[12,66] Although pain serves a protective function to warn the body of damage and to seek protection, untreated surgical pain and stress slow healing.[88] For example, postoperative pain in human patients predicts longer length of hospital stay, delayed ambulation, and long-term functional impairment.[95] Surgical pain in elderly human patients has been linked to poor quality of life, reduced wellbeing, physical disability and mortality.[71,88,121] Similarly, untreated pain in rodents may lead to reduced food and water intake, disrupted sleep, and changes in activity and species-specific behaviors, including grooming, nesting and burrowing.[66-68,83] Without effective analgesia, healing can be delayed. In humans, greater surgical pain is associated with slower healing of experimental wounds.[88] For orthopedic procedures, effective pain control is vital to promote continued limb usage to stimulate bone healing.[66,120] In Sprague–Dawley rats, mechanical loading is essential to femoral bone healing.[120,139,140,141] Without adequate analgesia, continued limb usage and the mechanical stress required for proper healing is hampered.[120] Analgesia will reduce pain, but not eliminate all painful sensations, therefore, providing analgesia is unlikely to result in overuse injury.

An effective way to reduce the stress and pain response to surgery is to provide preemptive and/or multimodal analgesia. For example, rats treated with morphine 30-min prior to surgery showed reduced postsurgical increases in plasma corticosterone.[107] Because pain pathways are complex, a multimodal analgesia plan is most likely to offer effective pain management. However, analgesic choice must be carefully considered as the use of some analgesics may influence study outcomes. For example, a study evaluating bone healing may need to avoid the use of NSAID as they are implicated in impairing the process of healing. Here we review the most commonly used analgesics in mice and rats including opioids, nonsteroidal antiinflammatory drugs (NSAID), local anesthetics, gabapentin, dissociative agents (ketamine), and acetaminophen, with particular application to surgical models of wound healing and orthopedics research.

Impact of analgesic choice on surgical orthopedic models. ***Opioids.*** Opioids are the mainstay of pain control for human patients.[38] Opioids provide effective analgesia, however, the abundance of opioid receptors throughout the body means the potential for side effects is significant. These include sedation, respiratory depression, bradycardia, emesis, constipation, urinary retention, cognitive impairment, addiction, and tolerance in humans.[9,13] Buprenorphine is most commonly used opioid for rodent models.[29,55] Benefits of buprenorphine administration include its low toxicity, minimal effects on the immune system, and compared with other opioids, minimal respiratory depression.[55]

The specific impact of opioids on rodent orthopedic models is still uncertain. Buprenorphine administration was shown to have a positive impact on tendon-to-bone healing in a gait analysis and tendon-bone healing ratio model.[23] Rats treated with buprenorphine (0.05 mg/kg SC, every 8 to 12 h, for 5 d) showed the greatest improvement as interpreted by gait analysis, limb loading, speed and force.[23] Others have confirmed the role of opioid receptors in the regulation of bone remodeling processes in rats. This was demonstrated by improved mechanical properties of bone following a 4 wk administration of either morphine (20 mg/kg/day SC) or buprenorphine hydrochloride (0.05 mg/kg/day SC) administration for 28 d in a model of osteoporosis.[64] Orthopedic models often result in chronic pain, which requires extended analgesia. In Sprague–Dawley rats, left femoral diaphysis fracture pain (where no analgesia was provided) was highest on day 1 postfracture; significantly decreased after 7 d; and returned to baseline (no pain) at 14 to 21 d.[50] Sustained-release buprenorphine (1.2 mg/kg, SC) provided analgesia for 2 to 3 d in a tibial defect model in male Sprague–Dawley rats, however, any secondary effect on the skeletal system was not specifically evaluated.[48] In humans, chronic opioid administration (including codeine, oxycodone, propoxyphene, or tramadol for greater than 3 mo) led to analgesic tolerance, a progressive lack of response to the drug.[42] Opioid tolerance may also lead to hyperalgesia, a neural sensitization in which opioids can sometimes cause increased pain.[75,114] In addition, chronic opioid administration may have adverse effects on bone healing. In a rat femoral fracture model, chronic morphine administration (5 mg/100 µL/kg every 8 h for 8 wk) decreased fracture callus remodeling and resorption.[27] Taken together, the available data suggest that a short course (2 to 3 wk) of opioid analgesia (namely buprenorphine) will not negatively impact bone healing. Because of buprenorphine's high-therapeutic index, analgesic effectiveness, and minimal negative effects on experimental outcomes in rodent orthopedic models, the authors recommend this analgesic as the primary analgesic choice for rodent orthopedic models.

Tramadol is an opioid agonist that functions by inhibiting serotonin and noradrenaline reuptake at the spinal cord. It may

be used as either an alternative to or adjunct to the aforementioned opioid analgesics. Both oral and injectable formulations are available.[34,47] Tramadol (10 mg/kg, IP) and gabapentin (80 mg/kg, SC) administered every 8 to 12 h for 5 d was found to provide a good alternative to other analgesics postoperatively in a rat rotator cuff tendon-to-bone healing model, however the analgesic effect is not as good as with an opioid such as buprenorphine (0.05 mg/kg SC every 8 to 12 h for 5 d).[23]

Little published information is available regarding the effect of tramadol on bone healing. Tramadol did not significantly inhibit human osteoblast activity in vitro.[51] Furthermore, bone healing was not significantly impacted by low (5 mg/kg) or high (20 mg/kg) doses of tramadol subcutaneously daily for 6 wk in Sprague–Dawley rats.[142]

Nonsteroidal antiinflammatory drugs (NSAID) and steroids. NSAID are valuable as a component of a multimodal analgesic regime. Physicians view NSAID as the safest, most effective way to treat postoperative pain.[85] Although NSAID are effective at reducing musculoskeletal pain, scientists may be hesitant to use this class of analgesics because of their potential to impact bone healing.[32,110] NSAID reduce pain by blocking cyclooxygenase (COX) enzymes (COX1 or COX2), leading to inhibition of the production of PGE_2, an inflammation-moderating prostaglandin. Selective PGE_2 agonists have been shown to speed up bone healing.[19,108] Multiple reviews have evaluated the effects of NSAID on bone healing without achieving a general consensus regarding its effects.[110] For example, animal studies have indicated the following nonselective and selective COX inhibitors result in impaired bone healing: acetylsalicylic acid (ASA),[3] ibuprofen,[4] ketoprofen,[86] etodolac,[41] meloxicam[113] and diclofenac.[78] Others demonstrate no effect on bone healing after administration of: ibuprofen,[96] ketoprofen[129] or diclofenac.[128] A third group of studies showed no effect on bone healing with short-term use of either ketoprofen[100] or diclofenac.[76,110] Further experimental studies (see Table 1) suggest that the effect of NSAID depends on the drugs themselves (that is dosages, dosing frequency, time of studies either early stages or later stages of bone healing); the specific animal models used: types and locations of bone fracture; the species, strains, ages, sexes, and numbers of animals used in each study; and the measurement methods and duration of the end-point of studies.[10,32]

Nonselective COX inhibitors. Studies in mice suggest that nonselective COX inhibitors can negatively impact orthopedic healing (see Table 1). Indomethacin (2 mg/kg/day PO) and ibuprofen (30 mg/kg/day PO) were reported to inhibit fracture healing when administered either short- (less than 1 wk) or long-term (4 to 8 wk).[16] Although ibuprofen (20 mg/kg PO administered preoperatively and every 8 to 12 h for 5 d after surgery) provided adequate early-stage postoperative analgesia, when compared to buprenorphine (0.05 mg/kg SC every 8 to 12 h for 5 d after surgery) and tramadol (10 mg/kg IP every 8 to 12 h for 5 d after surgery), it was found to impair bone healing in rats.[23] Ketorolac (4 mg/kg PO every 24 h for 21 d) was found to delay femoral fracture healing in male Sprague–Dawley rats more than parecoxib (1.5 mg/kg PO every 24 h for 21 d), a selective COX2 inhibitor.[53] Indomethacin (3 mg/kg SC for 7 wk) delayed spinal fusion in rats.[36] In a rat femur osteotomy model, short-term administration of indomethacin (200 mg/kg PO daily for 3 d) negatively impacted bone strength.[60] Because of the significant impact nonselective COX inhibitors had on bone healing, these drugs are not recommended for analgesia in orthopedic models.

Selective COX2 inhibitors. Long-term use of COX2 selective NSAID negatively impacts orthopedic models. In rats, diclofenac (5 mg/kg PO daily for 10 d) reduced osteoblast proliferation and migration, delaying bone defect healing at the distal femur.[76] Carprofen (2.2 mg/kg BID for 4 mo) inhibited bone healing in a canine model of tibial osteotomy.[103] Rofecoxib administration reduced blood flow and impaired fracture healing in a mouse transverse middiaphyseal femoral fracture model (5 mg/kg PO for 32 d)[97] and rabbit fibula osteotomy model (12.5 mg PO daily for 28 d).[101] In contrast, short-term administration of COX2 selective NSAID does not have a significant impact. Meloxicam (0.2 mg/kg/d IM for 10 d) impaired the fibular fracture healing process in Sprague–Dawley rats, yet the authors concluded there was not sufficient evidence of healing impairment to discourage use.[61] In rats, celecoxib (2 mg/kg/day PO for 5 d) reduced femur fracture callus mechanical strength during early stages of bone healing, but there was no significant effect when used prior to fracture or 2 wk after.[123] Another study in rabbits indicated that Rofecoxib (12.5 mg PO daily for 2 wk) did not interfere with bone ingrowth of the proximal medial tibial metaphysis when given during the initial or final 2 wk of treatment.[54] Taken together, these data suggest that COX2 selective NSAID may be useful as part of a short-term (that is, less than 10 d) multimodal analgesic regimen for orthopedic models. In the short-term, the benefits of adding NSAID as part of the analgesic plan outweighs any potential delays in healing.

Steroids. Steroid administration during fracture healing delays the process as steroids can decrease osteoblastic activity, growth factor production and matrix synthesis.[7,16,31] Long-term administration of prednisolone (0.15 mg/kg SC daily for 6 wk) inhibited bone healing in a rabbit ulnar osteotomy model.[135] Studies in rats[72] (prednisolone pellets 1.82 to 2.56 mg/kg/day SC for 90 d) and mice[132] (dexamethasone 2 to 4 mg/L in the drinking water for 12 wk) confirmed their susceptibility to glucocorticoid induced osteonecrosis. The effects of short-term steroid use are less well defined. In a rat femur osteotomy model, treatment with 3 consecutive injections of methylprednisolone (200 mg/kg IM daily for 3 d) had no significant effect on bone healing strength.[60] Because steroids have direct adverse effects on osteoblasts, osteoclasts, osteocytes and cartilage their use is not recommended for analgesia in orthopedic models.

Local anesthetics. Local anesthetics, (LA) can be used to provide analgesia to a specific area during intraoperative anesthesia and during the postoperative period. Local anesthetics diffuse across the plasma membrane and reversibly block voltage-gated sodium channels.[122] They are the only analgesic drug class that completely disrupts the transmission of nociception to the central nervous system. Local anesthesia offers the benefit of ease of application, simplicity and few side effects.[57] The effect of lidocaine or bupivacaine (0.3 mL of either 1% lidocaine with epinephrine, 1% lidocaine without epinephrine or 0.25% bupivacaine) was evaluated when administered at the fracture site in a rat femoral fracture model, and there were no differences in callus composition, tensile strength or histologic appearance during early fracture repair.[59] Based upon the available information, local anesthetics are a good addition as part of a multimodal analgesic regimen.

Impact of analgesic choice for wound healing models.

Opioids. Opioid receptors and ligands are a part of the skin's neuroendocrine system and opioids are widely used to control wound pain.[15,105] Many cells within the dermis are known to have opioid receptors. Keratinocytes, fibroblasts, neurons, and melanocytes are all known to express δ, μ and κ receptors. In early phase of wound healing, endogenous opioids are released

Table 1. Summary of cited studies evaluating the impact of analgesics used in rats or mice on orthopedic models.

Drugs	Dose, route, frequency	Strain/Species/Sex	Models	Effect on Model	Ref
		Opioids			
Buprenorphine	0.05 mg/kg SC every 8-12 h for 5 d	Sprague–Dawley rats; male	Rotator cuff tendon-to-bone healing	No effect	Caro and colleagues[23]
Morphine or Buprenorphine	20 mg/kg/day SC 0.05mg/kg/day SC for 28 d	Wistar rats; female	Osteoporotic changes	Positive	Janas and colleagues[64]
Morphine	5mg/100µL/kg every 8h for 8 wk	Sprague–Dawley rats; unknown sex	Femur diaphyseal fracture model	Negative (decreased callus strength)	Chrastil and colleagues[27]
Tramadol	Tramadol low (5 mg/kg) or high (20 mg/kg) dose SC daily for 6 wk	Sprague–Dawley rats; female	Femur fracture model	No effect	Caro and colleagues[23]
		Nonselective NSAID			
Indomethacin or Ibuprofen	2 mg/kg/day *PO* or 30 mg/kg/day *PO* for 1 or 8 wk	Mice; unknown sex	Femur fracture model	Negative	Boursinos and colleagues[16]
Ibuprofen	20 mg/kg *PO* every 8 to 12 for 5 d	Sprague–Dawley rats; male	Rotator cuff tendon-to-bone healing	Negative	Caro and colleagues[23]
Ketorolac	4 mg/kg *PO* every 24 h for 21 d	Sprague–Dawley rats; male	Femur fracture model	Negative	Gerstenfeld and colleagues[52]
		Selective NSAID			
Parecoxib	1.5 mg/kg *PO* every 24 h for 21 d	Sprague–Dawley rats; male	Femur fracture model	No effect	Gerstenfeld and colleagues[52]
Diclofenac	5 mg/kg *PO* daily for 10 d	Wistar rats; male	Femur defect model	Negative	Krischak and colleagues[76]
Rofecoxib	5 mg/kg *PO* for 32 d	Mice; unknown sex	Femur fracture model	Negative	Murnaghan and colleagues[97]
Celecoxib	2 mg/kg/day *PO* for 5 d	Sprague–Dawley rats; unknown sex	Femur fracture Model	No effect	Simon and O'Connor[123]
Meloxicam	0.2 mg/kg/d IM for 10 d	Sprague–Dawley rats; male	Fibular fracture model	Negative	Inal and colleagues[61]
		Steroids			
Prednisone	Pellets 1.82-2.56 mg/kg/day SC for 90 d	5 different inbred strains of rats; unknown sex	Glucocorticoid induced osteonecrosis	Negative	Kerachian and colleagues[72]
Dexamethasone	2-4 mg/L in the drinking water for 12 wk	BALB/cJ mice; unknown sex	Glucocorticoid induced osteonecrosis	Negative	Yang and colleagues[138]
Methyl-prednisolone	200 mg/kg IM daily for 3 d	Rats; unknown sex	Femur osteotomy	No effect	Hogevold and colleagues[60]
		Local Anesthetics			
Lidocaine or Bupivacaine	0.3 mL of lidocaine or bupivacaine (1% lidocaine with epinephrine, 1% lidocaine without epinephrine or 0.25% bupivacaine)	Long–Evans rats; male	Femur fracture model	No effect	Henry and colleagues[58]

Notes: negative generally indicates impairment of healing and repair, while positive generally indicates promotion of healing and repair.

from inflammatory cells at the site of injury and have been found to promote ischemic wound healing.[134]

Apart from their analgesic properties, opioid agonists modulate angiogenesis, a critical process for wound healing, while opioid antagonists (for example, naltrexone) have antiangiogenic effects.[105] A study in mice indicated that the µ-opioid receptor plays a critical role in the proliferation phase of wound healing and in granulation tissue formation.[134] In rats, topical administration BID for 7 d of an opioid preparation (fentanyl, 5 µg/g, hydromorphone, 0.2 mg/g or morphine, 1.5 mg/g) blended with a hydrocream base hastened midline wound closure by increasing granulation tissue and collagen formation, epidermal and dermal organization, and angiogenesis.[109] In an in vitro wound healing model, topical opioid administration via addition to the cell media for 48 h (including morphine, hydropmorphone, fentanyl, and buprenorphine) accelerated wound closure.[137] However, long-term high-doses of morphine (20 mg/kg IP daily for 14 d) delayed vascularization and closure

of excisional skin injury in mice.[80] Thus, because of the potential delay of wound healing with sustained use of buprenorphine (greater than 10 d), the authors recommend only short-term use of buprenorphine for acute surgical wound models.

Tramadol used as a local infiltration at the surgical site (1 mL of 2.5% tramadol) did not have adverse effects on wound healing in Wistar Albino rats.[106] Infiltration with tramadol (3 mL of 5% tramadol) reduced postoperative pain and did not affect wound repair.[57] Consequently, tramadol can be used for wound infiltration anesthesia without impacting the wound healing process.

NSAID and steroids. Few studies have evaluated the effect of NSAID on wound healing. Proper wound healing requires hemostasis, inflammation, proliferation, and remodeling.[56,99] NSAID have antiproliferative effects on vasculature and skin and can increase scarring and delay wound healing due to suppression of PGE_2.[56] A study in rats indicated that flunixin meglumine (1.1 mg/kg IM every 12 h for 14 d), reduced the inflammatory stage, but not the proliferative stage (when fibroplasia is a factor in eventual wound strength). Histologically, there were no differences in wound healing and strength between flunixin and nonflunixin treated groups.[38] Results are conflicting regarding the effects of COX2 selective NSAID on wound healing. A study in rats found that diclofenac (5 mg/kg PO twice daily for 10 d), reduced the number of fibroblasts and weakened epithelialization, although wound healing was not affected based upon clinical parameters.[76] Celecoxib administered in the diet (celecoxib 1500 ppm added to AIN-76A diet for 1 wk), was shown to delay wound healing in mice, but improve healing of pressure ulcers (5 mg/kg daily in water for 2 wk).[45,117] Overall, studies of the direct effects of NSAID on wound healing are inconclusive (see Table 2); however, because of the benefits of analgesia we recommend short-term (less than 1 wk) use of NSAID for analgesia in wound healing models.

Steroids. Steroids are recognized to inhibit wound healing through antiinflammatory effects and suppression of cellular responses, including fibroblast proliferation and collagen synthesis. Steroids also suppress genes responsible for interleukin signaling, keratinocyte proliferation, and cytoskeleton remodeling, and inhibiting the immune system, thus increasing the risk of infection.[126] Methylprednisolone (single dose, 40 mg/kg IM), a long-acting synthetic steroid, was shown to suppress wound healing in male CD1 mice[45] and male Sprague–Dawley rats (single dose of methylprednisolone acetate, 6 mg SC).[136] Other work in various species has similarly demonstrated up to 30% reduction in wound tensile strength with corticosteroid dosages of 15 to 40 mg/kg/day.[133] Topical steroids also inhibit acute wound healing. For example, weak-strength ((hydrocortisone cream 1%) and medium-strength (fluocinolone acetonide ointment 0.025%)) steroid preparations were reported to inhibit acute healing in hamsters.[84] Taken together, these data suggest that systemic and topical steroid use should be avoided in wound healing models (see Table 2).

Local anesthetics. Infiltration of a local anesthetic agent into the wound site can be an effective way to reduce postoperative pain.[115] LA affects the first 2 stages of wound healing, the inflammatory and proliferation stages.[17] LA offer antiinflammatory effects by reducing eicosanoid, prostaglandins, thromboxanes, leukotrienes, histamine, inflammatory cytokines and the scavenging of oxygen free radicals.[24] LA also suppress synthesis of mucopolysaccharides; reduce collagenization; and reduce the quantity of mast cells at the wound site.[94,116]

The data on the effects of local anesthesia on wound healing and breaking strength are conflicting (see Table 2).[63,132] Some studies indicate that lidocaine and bupivacaine inhibit collagen synthesis, cause cytotoxic effects, delay wound healing, and reduce wound breaking strength and collagen production.[40,44,57] A single infiltration dose of lidocaine did not significantly affect wound breaking strength in rabbits (0.5% lidocaine SC)[131] or rats (2% lidocaine, 2.5 mL injected in 4 different locations, SC).[37] However, another study in Sprague–Dawley rats reported that lidocaine infiltration (single infiltration, 2 mL of 0.5% to 2% lidocaine) impaired would breaking strength at 5 to 7 d in a dose-dependent manner that was potentiated by epinephrine.[94] In that study, tensile strength was also impaired by injection of water, suggesting that some impaired healing was due to the physical damage resulting from injection of fluid into the skin and subcutaneous tissue.[94] Another study demonstrated a single 3 mL subcutaneous infiltration of either lidocaine or prilocaine had no effect on wound healing in Sprague–Dawley rats, although a similar dose of either bupivacaine or levobupivacaine negatively affected wound tensile strength at 21 d.[73] These results suggest that bupivacaine and levobupivacaine negatively affect late-stage wound healing, and lidocaine or prilocaine have no effect on late-stage wound healing. These studies indicate that the effect of the LA on wound healing is dependent on the concentration, volume, duration and anesthetic classification (see Table 2).

EMLA cream, an emulsion of lidocaine and prilocaine, is a commonly used topical anesthetic. Results from a study in guinea pigs indicated that application of 0.25 mL of EMLA cream resulted in an increased inflammatory response, and was associated with susceptibility to wound infection.[111] A more recent study in mice indicated that twice-daily topical EMLA application had a beneficial effect on experimental wound healing and resulted in increased tensile strength of the wound tissues.[42]

Prolonged use of LA (for example via a wound catheter, a simple fenestrated tube placed at a wound for the purpose of continuous or intermittent administration of local anesthetics) or high LA concentrations should be used cautiously. The majority of studies regarding the effect of LA on wound healing were done at early stages of wound healing. It is unknown if there are lasting effects of LA on late-stage wound healing (that is, after remodeling). Liposomal bupivacaine is a prolonged-release formulation of bupivacaine HCl indicated for single-dose infiltration into the surgical site to produce postsurgical analgesia.[1] Our group did not see any significant impact on wound healing when used at 6 mg/kg SC in Sprague–Dawley rats.[69] In human patients, liposomal bupivacaine administered locally at the surgical wound site resulted in no clinically evident impact on wound or bone healing.[11] Due to the benefits of LA as a form of analgesia with minimal adverse effects, we recommend short-term (provided before or at the time of wound treatment) LA as acceptable in wound healing models.

Other analgesics for orthopedic and wound healing models.

Gabapentin. Gabapentin (80 mg/kg IP) has been used as an adjunctive analgesic to tramadol (10 mg/kg IP) in rodents.[90] Gabapentin (1200 mg/day, oral gavage twice daily) negatively affected bone fracture healing in a model using female Wistar Albino rats.[125] However, gabapentin (20 mg/kg oral gavage 1 d before surgery and once and day for 9 d postsurgery) minimally affected wound healing in in male Wistar Albino rats.[118] These data support the use of gabapentin in wound healing but not orthopedic surgical models.[118]

Ketamine. Ketamine (low dose) can be used as a preventative analgesic against hypersensitivity because of its action as a NMDA antagonist.[47] Pretreatment with ketamine (10 mg/kg SC) was reported to attenuate morphine-induced hyperalgesia

Table 2. Summary of cited studies evaluating the impact of analgesics used in rats or mice on wound healing models.

Drugs	Dose, route, frequency	Strain/Species/Sex	Models	Effect on Model	Ref
		Opioids			
Fentanyl,	Fentanyl (5 µg/g) twice daily	Fischer 344 rats; unknown sex	Wound healing	Positive	Poonwala and colleagues[109]
Hydromorphone, or	(0.2 mg/g) twice daily or				
Morphine	1.5 mg/g twice daily				
Morphine	20 mg/kg IP daily for 14 d	C57BL/6J mice; unknown sex	Excisional skin injury	Negative	Lam and colleagues[80]
Tramadol	1 mL of 2.5% tramadol as local infiltration	Wistar albino rats; unknown sex	Wound healing	No effect	Ozkan and colleagues[106]
		Nonselective NSAID			
Flunixin meglumine	1.1 mg/kg IM every 12 h for 14 d	Sprague–Dawley rats; male	Wound healing	No effect	Donner and colleagues[38]
Diclofenac	5 mg/kg *PO* twice daily for 10 d	Wistar rats; male	Wound healing	No effect	Krischak and colleagues[77]
		Selective NSAID			
Celecoxib	1500 ppm added to AIN-76A diet for 1 wk	C57BL6/J mice; unknown sex	Wound healing	Negative	Fairweather and colleagues[43]
Celecoxib	5 mg/kg daily in water for 2 wk	Swiss mice; male	Pressure ulcers	Positive	Romana-Souza and colleagues[117]
		Steroids			
Methyl-prednisolone	40 mg/kg IM once	CD1 mice; male	Wound healing	Negative	Fesser and colleagues[45]
Methyl-prednisolone	6 mg SC once	Sprague–Dawley rats; male	Wound healing	Negative	Wicke and colleagues[136]
		Local Anesthetics			
Lidocaine	2% SC	F344 rats; unknown sex	Wound healing	No effect	Dogan and colleagues[37]
Lidocaine	2 mL of 0.5% to 2%	Rats; unknown sex	Wound healing	Negative	Morris and Tracy[94]
Lidocaine or	lidocaine 7 mg/kg local infiltration	Sprague–Dawley rats; unknown sex	Wound healing	No effect	Kesici and colleagues[73]
Prilocaine	2 mg/kg local infiltration				
Bupivacaine or	2 mg/kg local infiltration or	Sprague–Dawley rats; unknown sex	Wound healing	Negative	Kesici and colleagues[73]
Levobupivacaine	2.5 mg/kg local infiltration				

Notes: negative generally indicates impairment of healing and repair, while positive generally indicates promotion of healing and repair.

in mouse bone fracture models.[93] When ketamine is used as a part of the rodent anesthesia plan, the subsequent surgical pain is reduced.[47] However, we were unable to find any published studies evaluating the impact of ketamine on wound healing.

Acetaminophen (Paracetamol/Tylenol). Acetaminophen is used as an oral analgesic in dogs and small mammals.[35,47,87] Acetaminophen did not negatively affect bone fracture healing in Sprague–Dawley rats (6 to 300 mg/kg PO for 10 d)[13,48] or strength of healing of the intestinal or abdominal fascia (anastomotic and abdominal wall healing model) in male Wistar rats (50 to 200 mg/kg/d PO for 3 to 7 d).[130] Others have described a lack of any effect on wound healing strength in a rat patellar tendon resection model when animals were administered acetaminophen (60 mg/kg for 7 d in the diet).[46] Acetaminophen can be used as an adjunct analgesic for orthopedic or wound healing surgical models.

Multimodal analgesia. Despite the known benefits of multimodal analgesia, few reports evaluate its use.[21,127] To our knowledge, data are not available that specifically evaluate the effects of a multimodal analgesic regime on either bone or wound healing in rodents. One study used a combination of tramadol (10 mg/kg IP, q 8 to 12 h for 5 d) and gabapentin in a male Sprague–Dawley rat tendon-to-bone healing model.[22] The study found no significant differences in healing, and the multimodal regimen was less efficacious in controlling postsurgical pain than was buprenorphine (0.05 mg/kg SC q 8 to 12 h for 5 d) or ibuprofen (10 mg/kg IP). Using other multimodal analgesic regimes (that is buprenorphine in combination with NSAID and/or local anesthetics) will increase the safety and efficacy of analgesia.[98] Given these data, we propose that best practice is to provide multimodal analgesia during rodent surgery.

Conclusion

Refinement of the analgesic plan. The majority of orthopedic and wound healing models cause moderate to severe pain. A

Table 3. The below table summarizes the authors' recommendations regarding the impact of analgesic choice on the surgical model

	Opioids	NSAID	Steroids	LA	Other
Orthopedic models—bone (fracture)	Recommend (buprenorphine, tramadol or local infiltration of tramadol)	Use caution (may consider short-term selective COX2 inhibitors)	Avoid	Recommended	Consider adjunctive analgesia with tramadol, ketamine, acetaminophen
Wound healing	Recommend (short-term buprenorphine; topical use of opioids)	Use caution (may consider short-term selective COX2 inhibitors)	Avoid	Use caution	Consider adjunctive analgesia tramadol, gabapentin, ketamine, acetaminophen

well-designed analgesic plan must be implemented by working closely with veterinarians familiar with the species being used. When possible, preemptive, multimodal analgesic techniques should be used to reduce the need for subsequent analgesic doses, speed recovery and wound healing, and to prevent chronic pain.

The analgesic choice depends on many factors: the dose, duration interval, route of administration, surgical model, species, strain, sex, and health. Other factors to consider are the skill of the surgeon and the amount of tissue trauma inherent in the model. Inexperienced surgeons may cause more tissue trauma than more skillful practitioners by applying excessive pressure or causing ischemia during hemostasis, which may require higher doses of pain relief or extended analgesic coverage. Training in proper surgical technique and anatomic knowledge relevant to the model studied is thus necessary to uphold the 3Rs principle of refinement.[98] IACUC must critically evaluate surgeon skill and possibly require additional training such as surgeon shadowing, practicing survival surgeries on mice that are euthanized immediately after procedures, and/or surgicaltraining on cadavers/inanimate models. Surgeons must consider how to reduce surgical time by working with a surgical technician or an anesthetist. The IACUC and veterinary staff must follow-up with researchers and work closely with them during surgical model development to ensure technical proficiency and animal welfare. Furthermore, if researchers will be responsible for postoperative care, they must be properly trained to detect pain/distress, evaluate animals for early euthanasia criteria and humane endpoints. Researchers must also understand how to contact the veterinary staff to request additional analgesia and supportive care as defined in the approved IACUC protocol. Regardless of the analgesia provided, non-pharmacologic treatment must always be considered as an adjunctive measure for analgesia in the postoperative period. Factors such as additional enrichment, social housing, soft bedding, nesting materials, and accessible and palatable food and water supplements may improve animal welfare, but should not replace chemical analgesia.

Concerns about the effects of analgesic use on experimental outcomes in both orthopedic and wound healing models may influence and possibly limit drug choice. Although full μ-opioids or NSAID can have negative effects on the orthopedic and wound healing models, limited information is available regarding other types of opioids (that is buprenorphine SR, butorphanol, or tramadol). Further evidence-based and risk-benefit ratio studies (especially with NSAID) are needed. In the authors' opinion, if NSAID are used in orthopedic models, short-term use of selective COX2 inhibitors and/or local anesthetics would be appropriate.[110] If infiltration of local anesthetics at the surgical site is contraindicated, researchers might consider using a regional nerve block using a LA (blocking the nerve proximal to surgical sites using a nerve locator). Prudent, short-term use of opioids or NSAID for pain associated with wound healing is appropriate, unless a clear scientific justification exists (see Table 3). No single universal analgesic plan can be applied uniformly to complicated surgical models, such as those used in orthopedic and wound healing research. Further studies designed to examine the efficacy and experimental effects of various analgesic regimens, in a variety of species and specific surgical models are needed for further refinement. In all cases, we stress the importance for veterinarians and researchers to work together closely to devise an optimal analgesic plan for the specific surgical model.

References

1. **Adamson TW, Kendall LV, Goss S, Grayson K, Touma C, Palme R, Chen JQ, Borowsky AD.** 2010. Assessment of carprofen and buprenorphine on recovery of mice after surgical removal of the mammary fat pad. J Am Assoc Lab Anim Sci **49:**610–616.
2. **Akil H, Mayer DJ, Liebeskind JC.** 1976. Antagonism of stimulation-produced analgesia by naloxone, a narcotic antagonist. Science **191:**961–962. https://doi.org/10.1126/science.1251210.
3. **Allen HL, Wase A, Bear WT.** 1980. Indomethacin and aspirin: effect of nonsteroidal anti-inflammatory agents on the rate of fracture repair in the rat. Acta Orthop Scand **51:**595–600. https://doi.org/10.3109/17453678008990848.
4. **Altman RD, Latta LL, Keer R, Renfree K, Hornicek FJ, Banovac K.** 1995. Effect of nonsteroidal antiinflammatory drugs on fracture healing: a laboratory study in rats. J Orthop Trauma **9:**392–400. https://doi.org/10.1097/00005131-199505000-00006.
5. **Amit Z, Galina ZH.** 1986. Stress-induced analgesia: adaptive pain suppression. Physiol Rev **66:**1091–1120. https://doi.org/10.1152/physrev.1986.66.4.1091.
6. **An YH, Friedman RJ, editors.** 1999. Animal models in orthopaedic research. Boca Raton (FL): CRC Press.
7. **Anderson K, Hamm RL.** 2014. Factors that impair wound healing. J Am Coll Clin Wound Spec **4:**84–91. https://doi.org/10.1016/j.jccw.2014.03.001.
8. **Ansell DM, Campbell L, Thomason HA, Brass A, Hardman MJ.** 2014. A statistical analysis of murine incisional and excisional acute wound models. Wound Repair Regen **22:**281–287. https://doi.org/10.1111/wrr.12148.
9. **Barletta JF, Asgeirsson T, Senagore AJ.** 2011. Influence of intravenous opioid dose on postoperative ileus. Ann Pharmacother **45:**916–923. https://doi.org/10.1345/aph.1Q041.
10. **Barry S.** 2010. Nonsteroidal antiinflammatory drugs inhibit bone healing: a review. Vet Comp Orthop Traumatol **23:**385–392. https://doi.org/10.3415/VCOT-10-01-0017.
11. **Baxter R, Bramlett K, Onel E, Daniels S.** 2013. Impact of local administration of liposome bupivacaine for postsurgical analgesia on wound healing: a review of data from 10 prospective, controlled clinical studies. Clin Ther **35:**312–320.e5. https://doi.org/10.1016/j.clinthera.2013.02.005.
12. **Bechert K, Abraham SE.** 2009. Pain management and wound care. J Am Col Certif Wound Spec **1:**65–71.
13. **Benyamin R, Trescot AM, Datta S, Buenaventura R, Adlaka R, Sehgal N, Glaser SE, Vallejo R.** 2008. Opioid complications and side effects. Pain Physician **11:**S105–S120.
14. **Bergenstock M, Min W, Simon AM, Sabatino C, O'Connor JP.** 2005. A comparison between the effects of acetaminophen

and celecoxib on bone fracture healing in rats. J Orthop Trauma **19:**717–723. https://doi.org/10.1097/01.bot.0000184144.98071.5d.

15. **Bigliardi PL, Dancik Y, Neumann C, Bigliardi-Qi M.** 2016. Opioids and skin homeostasis, regeneration and ageing— What's the evidence? Exp Dermatol **25:**586–591. https://doi.org/10.1111/exd.13021.
16. **Boursinos LA, Karachalios T, Poultsides L, Malizos KN.** 2009. Do steroids, conventional non-steroidal antiinflammatory drugs and selective Cox2 inhibitors adversely affect fracture healing? J Musculoskelet Neuronal Interact **9:**44–52.
17. **Brower MC, Johnson ME.** 2003. Adverse effects of local anesthetic infiltration on wound healing. Reg Anesth Pain Med **28:**233–240. https://doi.org/10.1097/00115550-200305000-00011.
18. **Butler RK, Finn DP.** 2009. Stress-induced analgesia. Prog Neurobiol **88:**184–202. https://doi.org/10.1016/j.pneurobio.2009.04.003.
19. **Cameron KO, Lefker BA, Ke HZ, Li M, Zawistoski MP, Tjoa CM, Wright AS, DeNinno SL, Paralkar VM, Owen TA, Yu L, Thompson DD.** 2009. Discovery of CP-533536: an EP2 receptor selective prostaglandin E2 (PGE2) agonist that induces local bone formation. Bioorg Med Chem Lett **19:**2075–2078. https://doi.org/10.1016/j.bmcl.2009.01.059.
20. **Carbone L.** 2011. Pain in laboratory animals: the ethical and regulatory imperatives. PLoS One **6:**1–6. https://doi.org/10.1371/journal.pone.0021578.
21. **Carbone L, Austin J.** 2016. Pain and laboratory animals: publication practices for better data reproducibility and better animal welfare. PLoS One **11:**1–24. https://doi.org/10.1371/journal.pone.0155001.
22. **Caro AC, Hankenson FC, Marx JO.** 2013. Comparison of thermoregulatory devices used during anesthesia of C57BL/6 mice and correlations between body temperature and physiologic parameters. J Am Assoc Lab Anim Sci **52:**577–583.
23. **Caro AC, Tucker JJ, Yannascoli SM, Dunkman AA, Thomas SJ, Soslowsky LJ.** 2014. Efficacy of various analgesics on shoulder function and rotator cuff tendon-to-bone healing in a rat (*Rattus norvegicus*) model. J Am Assoc Lab Anim Sci **53:**185–192.
24. **Cassuto J, Sinclair R, Bonderovic M.** 2006. Anti-inflammatory properties of local anesthetics and their present and potential clinical implications. Acta Anaesthesiol Scand **50:**265–282. https://doi.org/10.1111/j.1399-6576.2006.00936.x.
25. **Cevasco M, Ashley S, Cooper Z.** 2012. Chapter 44. Physiologic response to surgery. p 291–295. In: McKean SC, Ross JJ, Dressler DD, Brotman DJ, Ginsberg JS editors. Principles and practice of hospital medicine. New York (NY): The McGraw-Hill Companies.
26. **Cevasco M, Ashley S, Cooper Z, McKean SC, Ross JJ, Dressler DD, Brotman DJ, Ginsberg JS.** 2012. Principles and practice of hospital medicine. New York (NY): The McGraw-Hill Companies.
27. **Chrastil J, Sampson C, Jones KB, Higgins TF.** 2013. Postoperative opioid administration inhibits bone healing in an animal model. Clin Orthop Relat Res **471:**4076–4081. https://doi.org/10.1007/s11999-013-3232-z.
28. **Christian LM, Graham JE, Padgett DA, Glaser R, Kiecolt-Glaser JK.** 2007. Stress and wound healing. Neuroimmunomodulation **13:**337–346. https://doi.org/10.1159/000104862.
29. **Christoph T, Kögel B, Schiene K, Méen M, De Vry J, Friderichs E.** 2005. Broad analgesic profile of buprenorphine in rodent models of acute and chronic pain. Eur J Pharmacol **507:**87–98. https://doi.org/10.1016/j.ejphar.2004.11.052.
30. **US Office of Science and Technology Policy**. 1985. Laboratory animal welfare; US government principles for the utilization and care of vertebrate animals used in testing, research and training; notice. Fed Regist **50:**20864–20865.
31. **Cosman F, Nieves J, Herbert J, Shen V, Lindsay R.** 1994. High-dose glucocorticoids in multiple sclerosis patients exert direct effects on the kidney and skeleton. J Bone Miner Res **9:**1097–1105. https://doi.org/10.1002/jbmr.5650090718.
32. **Cottrell J, O'Connor JP.** 2010. Effect of non-steroidal anti-inflammatory drugs on bone healing. Pharmaceuticals (Basel) **3:**1668–1693. https://doi.org/10.3390/ph3051668.
33. **Desborough JP.** 2000. The stress response to trauma and surgery. Br J Anaesth **85:**109–117. https://doi.org/10.1093/bja/85.1.109.
34. **Desmeules JA, Piguet V, Collart L, Dayer P.** 1996. Contribution of monoaminergic modulation to the analgesic effect of tramadol. Br J Clin Pharmacol **41:**7–12. https://doi.org/10.1111/j.1365-2125.1996.tb00152.x.
35. **Dickinson AL, Leach MC, Flecknell PA.** 2009. The analgesic effects of oral paracetamol in two strains of mice undergoing vasectomy. Lab Anim **43:**357–361. https://doi.org/10.1258/la.2009.009005.
36. **Dimar JR 2nd, Ante WA, Zhang YP, Glassman SD.** 1996. The effects of nonsteroidal anti-inflammatory drugs on posterior spinal fusions in the rat. Spine **21:**1870–1876. https://doi.org/10.1097/00007632-199608150-00006.
37. **Doğan N, Uçok C, Korkmaz C, Uçok O, Karasu HA.** 2003. The effects of articaine hydrochloride on wound healing: an experimental study. J Oral Maxillofac Surg **61:**1467–1470.
38. **Donner GS, Ellison GW, Peyton LC, Crowley AM, Szempruch N, Williams JW.** 1986. Effect of flunixin meglumine on surgical wound strength and healing in the rat. Am J Vet Res **47:**2247–2251.
39. **Dorsett-Martin WA.** 2004. Rat models of skin wound healing: a review. Wound Repair Regen **12:**591–599. https://doi.org/10.1111/j.1067-1927.2004.12601.x.
40. **Drucker M, Cardenas E, Arizti P, Valenzuela A, Gamboa A.** 1998. Experimental studies on the effect of lidocaine on wound healing. World J Surg **22:**394–397, discussion 397–398. https://doi.org/10.1007/s002689900403.
41. **Endo K, Sairyo K, Komatsubara S, Sasa T, Egawa H, Ogawa T, Yonekura D, Murakami R, Yasui N.** 2005. Cyclooxygenase-2 inhibitor delays fracture healing in rats. Acta Orthop **76:**470–472. https://doi.org/10.1080/17453670510041439.
42. **Eroglu E, Eroglu F, Agalar F, Altuntas I, Sutcu R, Ozbasar D.** 2001. The effect of lidocaine/prilocaine cream on an experimental wound healing model. Eur J Emerg Med **8:**199–201.
43. **Fairweather M, Heit YI, Buie J, Rosenberg LM, Briggs A, Orgill DP, Bertagnolli MM.** 2015. Celecoxib inhibits early cutaneous wound healing. J Surg Res **194:**717–724. https://doi.org/10.1016/j.jss.2014.12.026.
44. **Fedder C, Beck-Schimmer B, Aguirre J, Hasler M, Roth-Z'graggen B, Urner M, Kalberer S, Schlicker A, Votta-Velis G, Bonvini JM, Graetz K, Borgeat A.** 2010. In vitro exposure of human fibroblasts to local anaesthetics impairs cell growth. Clin Exp Immunol **162:**280–288. https://doi.org/10.1111/j.1365-2249.2010.04252.x.
45. **Feeser VR, Menke NB, Ward KR, Loria RM, Diegelmann RF.** 2009. Androstenediol reverses steroid-inhibited wound healing. Wound Repair Regen **17:**758–761. https://doi.org/10.1111/j.1524-475X.2009.00528.x.
46. **Ferry ST, Dahners LE, Afshari HM, Weinhold PS.** 2007. The effects of common anti-inflammatory drugs on the healing rat patellar tendon. Am J Sports Med **35:**1326–1333. https://doi.org/10.1177/0363546507301584.
47. **Flecknell PA.** 2016. Laboratory animal anaesthesia, 4th ed. Waltham (MA): Elsevier.
48. **Foley PL, Liang H, Crichlow AR.** 2011. Evaluation of a sustained-release formulation of buprenorphine for analgesia in rats. J Am Assoc Lab Anim Sci **50:**198–204.
49. **Fracon RN, Teofilo JM, Moris IC, Lamano T.** 2010. Treatment with paracetamol, ketorolac or etoricoxib did not hinder alveolar bone healing: a histometric study in rats. J Appl Oral Sci **18:**630–634. http://dx.doi.org/10.1590/S1678-77572010000600016.
50. **Freeman KT, Koewler NJ, Jimenez-Andrade JM, Buus RJ, Herrera MB, Martin CD, Ghilardi JR, Kuskowski MA, Mantyh PW.** 2008. A fracture pain model in the rat: adaptation of a closed femur fracture model to study skeletal pain. Anesthesiology **108:**473–483. https://doi.org/10.1097/ALN.0b013e3181649351.
51. **Gerner P, O'Connor JP.** 2008. Impact of analgesia on bone fracture healing. Anesthesiology **108:**349–350. https://doi.org/10.1097/ALN.0b013e318164938c.
52. **Gerstenfeld LC, Einhorn TA.** 2004. COX inhibitors and their effects on bone healing. Expert Opin Drug Saf **3:**131–136. https://doi.org/10.1517/14740338.3.2.131.
53. **Gerstenfeld LC, Thiede M, Seibert K, Mielke C, Phippard D, Svagr B, Cullinane D, Einhorn TA.** 2003. Differential inhibition of fracture healing by non-selective and cyclooxygenase-2 selective non-steroidal anti-inflammatory drugs. J Orthop Res **21:**670–675. https://doi.org/10.1016/S0736-0266(03)00003-2.

54. **Goodman SB, Ma T, Mitsunaga L, Miyanishi K, Genovese MC, Smith RL.** 2005. Temporal effects of a COX-2-selective NSAID on bone ingrowth. J Biomed Mater Res A **72A:**279–287. https://doi.org/10.1002/jbm.a.30231.
55. **Guarnieri M, Brayton C, DeTolla L, Forbes-McBean N, Sarabia-Estrada R, Zadnik P.** 2012. Safety and efficacy of buprenorphine for analgesia in laboratory mice and rats. Lab Anim (NY) **41:**337–343. https://doi.org/10.1038/laban.152.
56. **Guo S, DiPietro LA.** 2010. Factors affecting wound healing. J Dent Res **89:**219–229. https://doi.org/10.1177/0022034509359125.
57. **Hanci V, Hakimoğlu S, Özaçmak H, Bektaş S, Özaçmak HS, Özdamar SO, Yurtlu S, Turan IÖ.** 2012. Comparison of the effects of bupivacaine, lidocaine, and tramadol infiltration on wound healing in rats. Rev Bras Anestesiol **62:**799–810. https://doi.org/10.1016/S0034-7094(12)70180-0.
58. **Hayes RL, Bennett GJ, Newlon PG, Mayer DJ.** 1978. Behavioral and physiological studies of non-narcotic analgesia in the rat elicited by certain environmental stimuli. Brain Res **155:**69–90. https://doi.org/10.1016/0006-8993(78)90306-2.
59. **Henry BJ, Kenison M, McVay C, Rolfe R, Graham S, Rasty J, Slauterbeck J, Dabezies EJ.** 2002. The effect of local hematoma blocks on early fracture healing. Orthopedics **25:**1259–1262.
60. **Høgevold HE, Grøgaard B, Reikerås O.** 1992. Effects of short-term treatment with corticosteroids and indomethacin on bone healing. A mechanical study of osteotomies in rats. Acta Orthop Scand **63:**607–611.
61. **Inal S, Kabay S, Cayci MK, Kuru HI, Altikat S, Akkas G, Deger A.** 2014. Comparison of the effects of dexketoprofen trometamol, meloxicam and diclofenac sodium on fibular fracture healing, kidney and liver: An experimental rat model. Injury **45:**494–500. https://doi.org/10.1016/j.injury.2013.10.002.
62. **Institute for Laboratory Animal Research.** 2011. Guide for the care and use of laboratory animals, 8th ed. Washington (DC): National Academies Press.
63. **Ireton JE, Unger JG, Rohrich RJ.** 2013. The role of wound healing and its everyday application in plastic surgery: a practical perspective and systematic review. Plast Reconstr Surg Glob Open **1:**e10–e19. https://doi.org/10.1097/GOX.0b013e31828ff9f4.
64. **Janas A, Folwarczna J.** 2017. Opioid receptor agonists may favorably affect bone mechanical properties in rats with estrogen deficiency-induced osteoporosis. Naunyn Schmiedebergs Arch Pharmacol **390:**175–185. https://doi.org/10.1007/s00210-016-1295-6.
65. **Jensen TS, Smith DF.** 1981. The role of consciousness in stress-induced analgesia. J Neural Transm **52:**55–60. https://doi.org/10.1007/BF01253097.
66. **Jirkof P.** 2017. Side effects of pain and analgesia in animal experimentation. Lab Anim (NY) **46:**123–128. https://doi.org/10.1038/laban.1216.
67. **Jirkof P, Cesarovic N, Rettich A, Nicholls F, Seifert B, Arras M.** 2010. Burrowing behavior as an indicator of post-laparotomy pain in mice. Front Behav Neurosci **4:**1–9. https://doi.org/10.3389/fnbeh.2010.00165.
68. **Jirkof P, Fleischmann T, Cesarovic N, Rettich A, Vogel J, Arras M.** 2013. Assessment of postsurgical distress and pain in laboratory mice by nest complexity scoring. Lab Anim **47:**153–161. https://doi.org/10.1177/0023677213475603.
69. **Kang SC, Jampachaisri K, Seymour TL, Felt SA, Pacharinsak C.** 2017. Use of liposomal bupivacaine for postoperative analgesia in an incisional pain model in rats (*Rattus norvegicus*). J Am Assoc Lab Anim Sci **56:**63–68.
70. **Kehlet H.** 1997. Multimodal approach to control postoperative pathophysiology and rehabilitation. Br J Anaesth **78:**606–617. https://doi.org/10.1093/bja/78.5.606.
71. **Kendig H, Browning CJ, Young AE.** 2000. Impacts of illness and disability on the well-being of older people. Disabil Rehabil **22:**15–22. https://doi.org/10.1080/096382800297088.
72. **Kerachian MA, Harvey EJ, Cournoyer D, Chow TY, Nahal A, Séguin C.** 2011. A rat model of early stage osteonecrosis induced by glucocorticoids. J Orthop Surg Res **6:**1–6. https://doi.org/10.1186/1749-799X-6-62.
73. **Kesici S, Kesici U, Ulusoy H, Erturkuner P, Turkmen A, Arda O.** 2018. [Effects of local anesthetics on wound healing]. Rev Bras Anestesiol **68:**375–382. https://doi.org/10.1016/j.bjan.2018.01.016. [Article in Portuguese].
74. **Kim DJ, Mustoe T, Clark RA.**2015. Cutaneous wound healing in aging small mammals: a systematic review. Wound Repair Regen **23:**318–339. https://doi.org/10.1111/wrr.12290.
75. **King T, Ossipov MH, Vanderah TW, Porreca F, Lai J.** 2005. Is paradoxical pain induced by sustained opioid exposure an underlying mechanism of opioid antinociceptive tolerance? Neurosignals **14:**194–205. https://doi.org/10.1159/000087658.
76. **Krischak GD, Augat P, Blakytny R, Claes L, Kinzl L, Beck A.** 2007. The non-steroidal anti-inflammatory drug diclofenac reduces appearance of osteoblasts in bone defect healing in rats. Arch Orthop Trauma Surg **127:**453–458. https://doi.org/10.1007/s00402-007-0288-9.
77. **Krischak GD, Augat P, Claes L, Kinzl L, Beck A.** 2007. The effects of non-steroidal anti-inflammatory drug application on incisional wound healing in rats. J Wound Care **16:**76–78. https://doi.org/10.12968/jowc.2007.16.2.27001.
78. **Krischak GD, Augat P, Sorg T, Blakytny R, Kinzl L, Claes L, Beck A.** 2007. Effects of diclofenac on periosteal callus maturation in osteotomy healing in an animal model. Arch Orthop Trauma Surg **127:**3–9. https://doi.org/10.1007/s00402-006-0202-x.
79. **Kumar V, Khan AA, Nagarajan K.** 2013. Animal models for the evaluation of wound healing activity. International bulletin of drug research.**3:**93–107.
80. **Lam CF, Chang PJ, Huang YS, Sung YH, Huang CC, Lin MW, Liu YC, Tsai YC.** 2008. Prolonged use of high-dose morphine impairs angiogenesis and mobilization of endothelial progenitor cells in mice. Anesth Analg **107:**686–692. https://doi.org/10.1213/ane.0b013e31817e6719.
81. **Lang A, Schulz A, Ellinghaus A, Schmidt-Bleek K.** 2016. Osteotomy models—the current status on pain scoring and management in small rodents. Lab Anim **50:**433–441. https://doi.org/10.1177/0023677216675007.
82. **Lennard TW, Shenton BK, Borzotta A, Donnelly PK, White M, Gerrie LM, Proud G, Taylor RM.** 1985. The influence of surgical operations on components of the human immune system. Br J Surg **72:**771–776. https://doi.org/10.1002/bjs.1800721002.
83. **Liles JH, Flecknell PA.** 1993. The effects of surgical stimulus on the rat and the influence of analgesic treatment. Br Vet J **149:**515–525. https://doi.org/10.1016/S0007-1935(05)80036-3.
84. **Marks JGJ, Cano C, Leitzel K, Lipton A.** 1983. Inhibition of wound healing by topical steroids. J Dermatol Surg Oncol **9:**819–821.
85. **Marquez-Lara A, Hutchinson ID, Nunez F Jr, Smith TL, Miller AN.** 2016. Nonsteroidal anti-inflammatory drugs and bone-healing: a systematic review of research quality. JBJS Rev **4:**1–14. doi: 10.2106/JBJS.RVW.O.00055
86. **Martins MV, da Silva MA, Medici Filho E, de Moraes LC, Castilho JC, da Rocha RF.** 2005. Evaluation of digital optical density of bone repair in rats medicated with ketoprofen. Braz Dent J **16:**207–212. https://doi.org/10.1590/S0103-64402005000300007.
87. **Matsumiya LC, Sorge RE, Sotocinal SG, Tabaka JM, Wieskopf JS, Zaloum A, King OD, Mogil JS.** 2012. Using the mouse grimace scale to reevaluate the efficacy of postoperative analgesics in laboratory mice. J Am Assoc Lab Anim Sci **51:**42–49.
88. **McGuire L, Heffner K, Glaser R, Needleman B, Malarkey W, Dickinson S, Lemeshow S, Cook C, Muscarella P, Melvin WS, Ellison EC, Kiecolt-Glaser JK.** 2006. Pain and wound healing in surgical patients. Ann Behav Med **31:**165–172. https://doi.org/10.1207/s15324796abm3102_8.
89. **McIrvine AJ, Mannick JA.** 1983. Lymphocyte function in the critically ill surgical patient. Surg Clin North Am **63:**245–261. https://doi.org/10.1016/S0039-6109(16)42980-4.
90. **McKeon GP, Pacharinsak C, Long CT, Howard AM, Jampachaisri K, Yeomans DC, Felt SA.** 2011. Analgesic effects of tramadol, tramadol-gabapentin, and buprenorphine in an incisional model of pain in rats (*Rattus norvegicus*). J Am Assoc Lab Anim Sci **50:**192–197.
91. **Meakins JL, Pietsch JB, Christou NV, Maclean LD.** 1980. Predicting surgical infection before the operation. World J Surg **4:**439–450. https://doi.org/10.1007/BF02393171.
92. **Minowada G, Welch WJ.** 1995. Clinical implications of the stress response. J Clin Invest **95:**3–12. https://doi.org/10.1172/JCI117655.

93. **Minville V, Fourcade O, Girolami JP, Tack I.** 2010. Opioid-induced hyperalgesia in a mice model of orthopaedic pain: preventive effect of ketamine. Br J Anaesth **104:**231–238. https://doi.org/10.1093/bja/aep363.
94. **Morris T, Tracey J.** 1977. Lignocaine: its effects on wound healing. Br J Surg **64:**902–903. https://doi.org/10.1002/bjs.1800641219.
95. **Morrison RS, Magaziner J, McLaughlin MA, Orosz G, Silberzweig SB, Koval KJ, Siu AL.** 2003. The impact of post-operative pain on outcomes following hip fracture. Pain **103:**303–311. https://doi.org/10.1016/S0304-3959(02)00458-X.
96. **Mullis BH, Copland ST, Weinhold PS, Miclau T, Lester GE, Bos GD.** 2006. Effect of COX-2 inhibitors and non-steroidal anti-inflammatory drugs on a mouse fracture model. Injury **37:**827–837. https://doi.org/10.1016/j.injury.2005.12.018.
97. **Murnaghan M, Li G, Marsh DR.** 2006. Nonsteroidal anti-inflammatory drug-induced fracture nonunion: an inhibition of angiogenesis? J Bone Joint Surg Am **88 Suppl 3:**140–147.
98. **National Research Council (US) Committee on on Recognition and Alleviation of Pain in Laboratory Animals**. 2009. Recognition and alleviation of pain in laboratory animals. Washington (DC): National Academies Press.
99. **Nguyen DT, Orgill DP, Murphy GF.** 2009. The pathophysiologic basis for wound healing and cutaneous regeneration, p 25–57. Chapter 4. In: Orgill DP, Blanco C. editors. Biomaterials for treating skin loss. Cambridge (United Kingdom): Woodhead Publishing.
100. **Nyangoga H, Aguado E, Goyenvalle E, Baslé MF, Chappard D.** 2010. A non-steroidal anti-inflammatory drug (ketoprofen) does not delay β-TCP bone graft healing. Acta Biomater **6:**3310–3317. https://doi.org/10.1016/j.actbio.2010.01.042.
101. **O'Connor JP, Capo JT, Tan V, Cottrell JA, Manigrasso MB, Bontempo N, Parsons JR.** 2009. A comparison of the effects of ibuprofen and rofecoxib on rabbit fibula osteotomy healing. Acta Orthop **80:**597–605. https://doi.org/10.3109/17453670903316769.
102. **O'Loughlin PF, Morr S, Bogunovic L, Kim AD, Park B, Lane JM.** 2008. Selection and development of preclinical models in fracture-healing research. J Bone Joint Surg Am **90a Suppl 1:**79–84. https://doi.org/10.2106/JBJS.G.01585.
103. **Ochi H, Hara Y, Asou Y, Harada Y, Nezu Y, Yogo T, Shinomiya K, Tagawa M.** 2011. Effects of long-term administration of carprofen on healing of a tibial osteotomy in dogs. Am J Vet Res **72:**634–641. https://doi.org/10.2460/ajvr.72.5.634.
104. **Ossipov MH, Dussor GO, Porreca F.** 2010. Central modulation of pain. J Clin Invest 120: 3779–3787. https://doi.org/10.1172/JCI43766.
105. **Ondrovics M, Hoelbl-Kovacic A, Fux DA.** 2017. Opioids: Modulators of angiogenesis in wound healing and cancer. Oncotarget **8:**25783–25796. https://doi.org/10.18632/oncotarget.15419.
106. **Özkan D, Seker D, Ergil J, Yalcindag A, Han U, Ginis Z, Akinci M, Delibas N.** 2013. The effects of tramadol infiltration on wound healing in rats. Acta Chir Belg **113:**434–438. https://doi.org/10.1080/00015458.2013.11680959.
107. **Page GG, McDonald JS, Ben-Eliyahu S.** 1998. Pre-operative versus postoperative administration of morphine: impact on the neuroendocrine, behavioural, and metastatic-enhancing effects of surgery. Br J Anaesth **81:**216–223. https://doi.org/10.1093/bja/81.2.216.
108. **Paralkar VM, Borovecki F, Ke HZ, Cameron KO, Lefker B, Grasser WA, Owen TA, Li M, DaSilva-Jardine P, Zhou M, Dunn RL, Dumont F, Korsmeyer R, Krasney P, Brown TA, Plowchalk D, Vukicevic S, Thompson DD.** 2003. An EP2 receptor-selective prostaglandin E2 agonist induces bone healing. Proc Natl Acad Sci USA **100:**6736–6740. https://doi.org/10.1073/pnas.1037343100.
109. **Poonawala T, Levay-Young BK, Hebbel RP, Gupta K.** 2005. Opioids heal ischemic wounds in the rat. Wound Repair Regen **13:**165–174. https://doi.org/10.1111/j.1067-1927.2005.130207.x.
110. **Pountos I, Georgouli T, Calori GM, Giannoudis PV.** 2012. Do nonsteroidal anti-inflammatory drugs affect bone healing? A critical analysis. ScientificWorldJournal **2012:**1–14. https://doi.org/10.1100/2012/606404.
111. **Powell DM, Rodeheaver GT, Foresman PA, Hankins CL, Bellian KT, Zimmer CA, Becker DG, Edlich RF.** 1991. Damage to tissue defenses by EMLA cream. J Emerg Med **9:**205–209. https://doi.org/10.1016/0736-4679(91)90414-B.
112. **Price DD.** 1996. Selective activation of A-delta and C nociceptive afferents by different parameters of nociceptive heat stimulation: a tool for analysis of central mechanisms of pain. Pain **68:**1–3.
113. **Ribeiro FV, César-Neto JB, Nociti FH Jr, Sallum EA, Sallum AW, De Toledo S, Casati MZ.** 2006. Selective cyclooxygenase-2 inhibitor may impair bone healing around titanium implants in rats. J Periodontol **77:**1731–1735. https://doi.org/10.1902/jop.2006.060119.
114. **Rivat C, Ballantyne J.** 2016. The dark side of opioids in pain management: basic science explains clinical observation. Pain Rep **1:**1–9. https://doi.org/10.1097/PR9.0000000000000570.
115. **Roberge CW, McEwen M.** 1998. The effects of local anesthetics on postoperative pain. AORN J **68:**1003–1012. https://doi.org/10.1016/S0001-2092(06)62134-0.
116. **Rodrigues FV, Hochman B, Wood VT, Simoes MJ, Juliano Y, Ferreira LM.** 2011. Effects of lidocaine with epinephrine or with buffer on wound healing in rat skin. Wound Repair Regen **19:**223–228. https://doi.org/10.1111/j.1524-475X.2010.00654.x.
117. **Romana-Souza B, Santos JS, Bandeira LG, Monte-Alto-Costa A.** 2016. Selective inhibition of COX-2 improves cutaneous wound healing of pressure ulcers in mice through reduction of iNOS expression. Life Sci **153:**82–92. https://doi.org/10.1016/j.lfs.2016.04.017.
118. **Sarıtaş TB, Korkmaz M, Sevimli A, Sarıtaş ZK.** 2016. Comparison of the effects of gabapentin and pregabalin on wound healing in rats. Int Wound J **13:**748–753. https://doi.org/10.1111/iwj.12364.
119. **Schmauss D, Weinzierl A, Schmauss V, Harder Y.** 2018. Common rodent flap models in experimental surgery.Eur Surg Res **59:**255–264. https://doi.org/10.1159/000492414.
120. **Schwarz C, Wulsten D, Ellinghaus A, Lienau J, Willie BM, Duda GN.** 2013. Mechanical load modulates the stimulatory effect of BMP2 in a rat nonunion model. Tissue Eng Part A **19:**247–254. https://doi.org/10.1089/ten.tea.2012.0265.
121. **Scudds RJ, Robertson J McD.** 1998. Empirical evidence of the association between the presence of musculoskeletal pain and physical disability in community-dwelling senior citizens. Pain **75:**229–235. https://doi.org/10.1016/S0304-3959(97)00224-8.
122. **Shah J, Votta-Velis EG, Borgeat A.** 2018. New local anesthetics. Best Pract Res Clin Anaesthesiol **32:**179–185. https://doi.org/10.1016/j.bpa.2018.06.010.
123. **Simon AM, O'Connor JP.** 2007. Dose and time-dependent effects of cyclooxygenase-2 inhibition on fracture-healing. J Bone Joint Surg Am **89:**500–511. https://doi.org/10.2106/JBJS.F.00127.
124. **Singer AJ, Clark RA.** 1999. Cutaneous wound healing. N Engl J Med **341:**738–746. https://doi.org/10.1056/NEJM199909023411006.
125. **Sofu H, Kockara N, Aydin BK, Suleyman B, Tayfur M, Malkoc I.** 2016. Should orthopedic surgeons consider the effects of gabapentin administration on bone healing while treating a long bone fracture: experimental study in a rat model. SICOT J **2:**1–6. https://doi.org/10.1051/sicotj/2016028.
126. **Stojadinovic O, Lee B, Vouthounis C, Vukelic S, Pastar I, Blumenberg M, Brem H, Tomic-Canic M.** 2007. Novel genomic effects of glucocorticoids in epidermal keratinocytes: inhibition of apoptosis, interferon-γ pathway, and wound healing along with promotion of terminal differentiation. J Biol Chem **282:**4021–4034. https://doi.org/10.1074/jbc.M606262200.
127. **Stokes EL, Flecknell PA, Richardson CA.** 2009. Reported analgesic and anaesthetic administration to rodents undergoing experimental surgical procedures. Lab Anim **43:**149–154. https://doi.org/10.1258/la.2008.008020.
128. **Tiseo BC, Namur GN, de Paula EJ, Junior RM, de Oliveira CR.** 2006. Experimental study of the action of COX-2 selective nonsteroidal anti-inflammatory drugs and traditional anti-inflammatory drugs in bone regeneration. Clinics (Sao Paulo) **61:**223–230. https://doi.org/10.1590/S1807-59322006000300007.
129. **Urrutia J, Mardones R, Quezada F.** 2007. The effect of ketoprophen on lumbar spinal fusion healing in a rabbit model. Laboratory investigation. J Neurosurg Spine **7:**631–636. https://doi.org/10.3171/SPI-07/12/631.
130. **van der Vijver RJ, van Laarhoven CJ, Lomme RM, Hendriks T.** 2012. Paracetamol does not compromise early wound repair in the

intestine or abdominal wall in the rat. Anesth Analg **115:**1451–1456. https://doi.org/10.1213/ANE.0b013e31826a4253.

131. **Vasseur PB, Paul HA, Dybdal N, Crumley L.** 1984. Effects of local anesthetics on healing of abdominal wounds in rabbits. Am J Vet Res **45:**2385–2388.
132. **Waite A, Gilliver SC, Masterson GR, Hardman MJ, Ashcroft GS.** 2010. Clinically relevant doses of lidocaine and bupivacaine do not impair cutaneous wound healing in mice. Br J Anaesth **104:**768–773. https://doi.org/10.1093/bja/aeq093.
133. **Wang AS, Armstrong EJ, Armstrong AW.** 2013. Corticosteroids and wound healing: clinical considerations in the perioperative period. Am J Surg **206:**410–417. https://doi.org/10.1016/j.amjsurg.2012.11.018.
134. **Wang Y, Gupta M, Poonawala T, Farooqui M, Li Y, Peng F, Rao S, Ansonoff M, Pintar JE, Gupta K.** 2017. Opioids and opioid receptors orchestrate wound repair. Transl Res **185:**13–23. https://doi.org/10.1016/j.trsl.2017.05.003.
135. **Waters RV, Gamradt SC, Asnis P, Vickery BH, Avnur Z, Hill E, Bostrom M.** 2000. Systemic corticosteroids inhibit bone healing in a rabbit ulnar osteotomy model. Acta Orthop Scand **71:**316–321. https://doi.org/10.1080/000164700317411951.
136. **Wicke C, Halliday B, Allen D, Roche NS, Scheuenstuhl H, Spencer MM, Roberts AB, Hunt TK**. 2000. Effects of steroids and retinoids on wound healing. Arch Surg **135:**1265–1270. https://doi.org/10.1001/archsurg.135.11.1265.
137. **Wolf NB, Küchler S, Radowski MR, Blaschke T, Kramer KD, Weindl G, Kleuser B, Haag R, Schäfer-Korting M.** 2009. Influences of opioids and nanoparticles on in vitro wound healing models. Eur J Pharm Biopharm **73:**34–42. https://doi.org/10.1016/j.ejpb.2009.03.009.
138. **Yang L, Boyd K, Kaste SC, Kamdem Kamdem L, Rahija RJ, Relling MV.** 2009. A mouse model for glucocorticoid-induced osteonecrosis: effect of a steroid holiday. J Orthop Res **27:**169–175. https://doi.org/10.1002/jor.20733.
139. **Yeomans DC, Cooper BY, Vierck CJ Jr.** 1996. Effects of systemic morphine on responses of primates to first or second pain sensations. Pain **66:** 253–263. https://doi.org/10.1016/0304-3959(96)03082-5.
140. **Yeomans DC, Pirec V, Proudfit HK.** 1996. Nociceptive responses to high and low rates of noxious cutaneous heating are mediated by different nociceptors in the rat: behavioral evidence. Pain **68:**133–140.
141. **Yeomans DC, Proudfit HK.** 1996. Nociceptive responses to high and low rates of noxious cutaneous heating are mediated by different nociceptors in the rat: electrophysiological evidence. Pain **68:**141–150.
142. **Zhang Chao Dr, Dong Y.** 2007. Study of tramadol hydrochloride's efficacy on the bone fracture healing process in SD rats. Eur J Pain Suppl **1:**103–104. https://doi.org/10.1016/S1754-3207(08)60035-8.