Non-Steroidal Anti-Inflammatory Drugs Chronotherapy may Enhance Bone Healing after Surgery

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June 2019

A thesis submitted to McGill University in partial fulfillment of the requirements for a Doctoral of Philosophy degree

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DEDICATION

TO MY WIFE BAN AND MY CHILDREN SAMA AND AHMED

Preface

This thesis work uses a manuscript-based format, following the guidelines of Graduate and Postdoctoral Studies of McGill University. Introduction, literature review, and methods chapters are followed by three manuscripts that present the methodological development and findings of the project. All the chapters were written by me (Haider Al-Waeli) under the supervision of Dr. Belinda Nicolau and Dr. Faleh Tamimi.

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ABSTRACT

Introduction: Effective postoperative pain control is an essential and humanitarian need for any surgical procedure, including bone fracture. Inadequate pain control may cause significant morbidity, impaired recovery and increased hospital costs. Postoperative pain is often managed with opioids; however, with the ongoing opioid epidemic, better therapeutic strategies are urgently needed. Non-steroidal anti-inflammatory drugs (NSAID) could be very useful for postoperative pain management. However, NSAID can impair bone healing outcome by influencing key inflammatory pathways.

One promising avenue for optimized pain management is rooted in the body's biological circadian rhythm. This thesis aims to contribute to gaps in the knowledge of understanding the effect of NSAIDs administration on bone healing and the effect of timing drug administration on this process.

Methods: To address these objectives, I used a combination of research methodologies. First, I conducted a systematic review and meta-analysis to estimate the effect of NSAIDs administration on fractured bone healing. Second, I comprised a series of experimental trials to test the hypothesis that the timing of NSAID administration affects postoperative fracture pain behavioural indices and bone healing measurements in a murine animal model. Also, these experiments aim to identify the inflammatory cytokines and metabolic pathways affected by the timing of NSAIDs administration. Finally, I developed a randomized clinical trial protocol to test the above hypothesis in human subjects.

Results: The findings from the meta-analysis of the published animal studies confirmed the negative effect of NSAIDs after bone fracture but suggested that the negative effects of NSAIDs

after bone fracture is different in mice compared to another animal model, in females compared to males and in younger compared with old animals. Our results from the quality risk assessment of the included studies demonstrated poorly reporting of randomization, blinding, allocation concealment and sample size calculation. In our experimental trials, we showed that NSAIDs' use is most effective in managing the pain and healing of bone fracture when they are administered during the active phase of the circadian rhythm in mice after bone fracture surgery. Limiting NSAIDs treatment to the active phase of the circadian rhythm were mediated by changes in the expression of >500 genes at the surgical site, including the clock gene Per2, and increases the serum level of anti-inflammatory cytokines interleukin-13 (IL-13), interleukin-4 (IL-4) and vascular endothelial growth factor (VEGF). Finally, we have prepared a protocol for a randomized controlled trial (RCT), which has been approved by the Ethical Review Board at the Jordan University of Science and Technology (Irbid, Jordan). The study is currently being conducted since May 2018.

Conclusion: Our systematic review and meta-analysis indicated the need to conduct more controlled and high-quality pre-clinical animal studies regarding the effect of NSAIDs on bone healing. Our preclinical trials using a murine tibia fracture surgical model demonstrated that NSAIDs are most effective in managing postoperative pain, healing and recovery when administrated during the active phase of the circadian rhythm. Our results, if confirmed in RCT in humans, may change clinical practice of all bone-related surgical interventions.

ABRÉGÉ

Introduction : un contrôle efficace de la douleur postopératoire constitue un aspect essentiel de toute intervention chirurgicale, et ce pour tous types de fractures y compris les fractures osseuses. Un contrôle insuffisant de la douleur postopératoire peut entraîner des taux de morbidité importante, une récupération difficile ainsi qu'une augmentation des coûts de la santé. . La douleur postopératoire est souvent contrôlée par l'utilisation d'opioïdes. Cependant, l'émergence d'une épidémie de surdoses d'opioïdes nécessite de nouvelles interventions thérapeutiques. Les traitements aux anti-inflammatoires non stéroïdiens (AINS) représentent une alternative intéressante pour la gestion de la douleur postopératoire. Toutefois, ces agents peuvent nuire aux processus de guérison osseuse en interférant sur les mécanismes principaux de l'inflammation.

L'étude des mécanismes circadiens pourrait fournir une piste prometteuse dans la recherche d'une gestion optimale de la douleur postopératoire. Les objectif principaux de cette thèse sont 1) d'identifier les lacunes dans les connaissances relatives à la compréhension de l'effet de l'administration d'AINS sur la guérison des os et 2) décrire les effets de différents temps d'administration du médicament sur le processus de guérison osseuse.

Méthodes : Pour atteindre ces objectifs, je propose d'utiliser une combinaison de méthodologies de recherche. En premier lieu, une revue systématique et une méta-analyse méthodologique a été complétée pour estimer l'effet de l'administration d'AINS sur la guérison des os fracturés. En second lieu, j'ai effectué une série d'expériences visant à vérifier l'hypothèse selon laquelle le temps d'administration d'AINS possède une influence sur les indices comportementaux de la douleur liée à une fracture postopératoire et sur les mesures de guérison des os chez un modèle

animal murin. De plus, ces expériences visent à identifier les cytokines pro-inflammatoires et les voies métaboliques affectées par le temps d'administration des AINS. Enfin, j'ai développé un protocole d'essais cliniques pour tester l'hypothèse ci-dessus chez des sujets humains.

Résultats : Les résultats de la méta-analyse des études animales publiées ont confirmé l'effet négatif des AINS sur la guérison d'une fracture osseuse. Cependant, ces mêmes études suggèrent que les effets négatifs des AINS sur la guérison osseuse sont différents 1) chez la souris par rapport à un autre modèle animal, 2) chez les femelles par rapport aux males et 3) chez les plus jeunes comparé aux vieux. L'évaluation de la qualité des études incluses m'a permis d'identifier ces lacunes méthodologiques: plusieurs d'entre-elles possèdent des défauts au niveau de la randomisation, de d'échantillonnage à l'aveugle, et de taille d'échantillon. Lors de nos essais précliniques sur des souris, nous avons démontré l'efficacité des AINS sur le traitement de la douleur et la guérison des fractures osseuses lorsque ceux-ci sont administré durant la phase active du rythme circadien. De plus, l'effet positif des AINS en phase active était accompagné de modifications de l'expression de >500 gènes au site chirurgical, dont le gène horloge Per2, et d'une augmentation du taux sérique de cytokines anti-inflammatoires tels l'interleukine-13 (IL-13), l'interleukine-4 (IL-4) et le facteur de croissance de l'endothélium vasculaire (VEGF). Enfin, nous avons préparé un protocole pour un essai contrôlé randomisé (ECR), qui a été approuvé par le comité d'éthique de l'Université des sciences et technologies de Jordan (Irbid, Jordan). L'étude a commencé en mai 2018 et la collecte de données est terminée. L'analyse des données est en cours.

Conclusion : Notre projet a mis en évidence la nécessité d'augmenter la qualité méthodologique des études précliniques qui évaluent les effets des AINS sur la guérison des fracture osseuses.

Nos essais précliniques ont démontré que dans un modèle chirurgical de fracture du tibia murin, les AINS sont les plus efficaces pour gérer la douleur, la guérison et le rétablissement postopératoires lorsque l'administration du médicament est limitée à la phase active du rythme circadien. Dans l'éventualité où ces résultats se confirment par nos essais contrôlés randomisés, ces résultats pourraient changer les traitements postopératoires de toutes les interventions chirurgicales liées aux os.

ACKNOWLEDGMENT

I do not know the words that can express how grateful I am to my supervisor Dr Belinda Nicolau, her majestic approach in training us as her students was something I will treasure all my life. She was the teacher, the colleague and before all that the mentor who is always available when you want a decision in your life. I am a better researcher, learner and before that a person because of her. Thank you, Dr Nicolau, for being our Angel during this journey.

Thank you for my other supervisor Dr Faleh Tamimi for being an amazing teacher who dedicated time and experience of reshaping an old man like me especially as a researcher and exposed me to all the skills available for making this journey of a better outcome. Dr. Tamimi was always available when I need to talk about the research or any other topic in life, as his knowledge is something that enriched mine during the last 5 years.

Thank you to Dr. Genevieve Castonguay with all her support, guidance and being around us in the lab when we need the advice and for making the atmosphere in the lab so lively. I am very gratitude to her also for her invaluable contribution to manuscripts editing.

Big Thank you to the members of my advisory committee Dr. Laura Stone, Dr Herenia Laurence and Dr. Raphael de Souza for all the guidance and support during my study and for providing the advice and the encouragement to continue in my study.

My sincere appreciation to the Oral Health and Society Research Division, McGill University, for being such colleagues and friends who were with me in my learning journey and I was so blessed to be with them learning from them and with them, they were amazing. Special thanks to Maria Palumbo, Student affairs coordinator, Faculty of Dentistry, for helping me since my application

as a Ph.D student to the daily emails of reminders about registration sand award and she never closed any door in our face even for simple question.

I wish to acknowledge the funding agencies of this project: Fonds de la recherche en santé du Québec (FRQS), Ontario Student Assistant Program (OSAP), and Alpha Omega Research Foundation Canada, for providing me with fellowships during my doctoral program.

STATEMENT OF ORIGINALITY

The work presented in this thesis is an original research. We are unaware of any previous studies that investigated the association of the timing of NSAID administration and surgery time with the healing of bone fractures. Circadian rhythm of inflammation and immunity along with the timing of delivering medication and performing the surgery seems to affect the healing outcomes and side effects. Also, clock genes have been involved in the bone healing fracture. Nevertheless, anti-inflammatory medications are necessary for the optimum recovery, but at the same time may delay bone healing. Therefore, my Ph.D project is to optimize already available therapeutic agents with broader implications and to further our understanding of NSAIDs' use in the healing of bone fractures. The project is an example of an interdisciplinary approach that involves systematic review and meta-analysis along with experimental animal trials ending with the preparation of randomized clinical trial in humans to test my hypothesis.

CONTRIBUTIONS of CO-AUTHORS

Manuscript I:

Non-Steroidal Anti-Inflammatory Drugs and Bone Healing in Animal Models – A Systematic Review and Meta-analysis

Under review in the Injury Journal

Haider Al-Waeli, Ph.D Candidate: conceived the topic of the manuscript and object of the analyses, performed all steps of the systematic review (searching the literature, selection of studies, data extraction and quality assessment), statistical analyses, and wrote the manuscript.

Ana Paula Reboucas, Research Associate, Faculty of Dentistry, Federal University of Minas Gerais, Brazil: participated in the search process, screening and selection of eligible studies.

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Belinda Nicolau, Associate Professor, Faculty of Dentistry, McGill University, Montreal, QC, Canada: Supervised the conception of the manuscript, and all steps of the systematic review and meta-analyses including the statistical analyses and writing the manuscript.

Manuscript II:

Non-Steroidal Anti-Inflammatory Drugs' Chronotherapy Could Enhance Healing of Fractured Bone in Mice

To be submitted to the Science Translational Medicine Journal

Haider Al-Waeli, Ph.D Candidate: conducted the in vivo experiments (animal handling, surgery, injection, bone harvesting, blood collection, mechanical bending assessment, RNA extract and analysis, Luminex cytokines analysis urine and blood collection, bioassays, locomotive activity and radiographic, mechanical and physical assessment of bone), statistical analysis, microarray gene expression analysis and submission to the Gene Expression Omnibus (GEO) database and drafted the manuscript.

Belinda Nicolau, Associate Professor, Faculty of Dentistry, McGill University, Montreal, QC, Canada: conceived the study, reviewed and analyzed the data, and edited the manuscript.

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Manuscript III:

Efficacy of Non-Steroidal Anti-Inflammatory Drug (Ibuprofen) Chronotherapy in Healing After Surgical Extraction of the Mandibular Third Molar – A Randomized Controlled Trial Protocol

Manuscript under preparation

Haider Al-Waeli, Ph.D Candidate: Provided the idea for the study, established the hypothesis, construct the database, submitted the protocol to clinicaltrial.gov and wrote and revised the manuscript.

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Faleh Tamimi, Associate Professor, Faculty of Dentistry, McGill University, Montreal, QC, Canada: conceived the study, participate inthe designed of the trial, and reviewed the manuscript.

Belinda Nicolau, Associate Professor, Faculty of Dentistry, McGill University, Montreal, QC, Canada: Supervised the conception of the manuscript, conceived the study, designed the trial, and edited and reviewed the manuscript.

1 Introduction

Bone fractures are extremely painful and can cause significant morbidity and even mortality in some cases. They account for 17.5% of emergency room injuries in Canada (1). There are over 100,000 non-union fractures every year in the USA alone, with expected Medicare costs of more than US$25 billion in 2025 (2, 3). Considering the social and financial overburden of bone loss and repair after injuries or surgeries, there is an urgent need to better understand the biological, cellular and molecular mechanisms of bone healing.

A significant issue in dealing with bone fracture surgeries (e.g., orthopedic or craniofacial surgeries) is the management of postoperative pain, particularly in patients who require immediate mechanical loading of the operated bone or joint (4). Currently, postoperative pain management in bone-related surgeries is limited to three classes of drugs: acetaminophen, opioids and nonsteroidal anti-inflammatory drugs (NSAID) (5, 6). However, these drugs have several significant limitations. Acetaminophen is ineffective on severe pain (5-8) and opioids may cause constipation and addiction (7, 8). Although largely used for postoperative pain control, NSAIDs (e.g., ibuprofen) may delay bone healing or increase bone non-union outcomes (9, 10). NSAIDs inhibit prostaglandin signalling, which can impair osteoclastic and osteoblastic activities and subsequently, bone healing (11). Substantial efforts have been made to develop NSAIDs that would selectively inhibit catabolic activities while sparing anabolic pathways. However, progress towards bone-related applications has been modest (12).

Bone metabolism and inflammation follow circadian rhythms regulated by clock genes (13). Accordingly, anti-inflammatory mediators and associated immune cells, and bone formation activity peak during sleep (resting phase), whereas pro-inflammatory mediators, osteoclast cells activity and bone resorption peek when animals are awake (active phase) (13, 14).

Therefore, timing NSAIDs administration with the peak of osteoclastic activity and the pro-inflammatory response after bone injury at the beginning of the active phase could maximize the benefit of the drugs while avoiding their negative effects on osteoblasts. While these are plausible mechanisms, there are no studies evaluating the effect of timing of NSAID administration on bone healing. My Ph.D work addresses this gap in the literature.

The overall aim of this thesis is to understand better the effect of the timing of NSAID administration on bone healing. We hypothesize that circadian variations in inflammation caused by clock genes influence both bone fracture healing and the effectiveness of NSAIDs therapy. Accordingly, limiting NSAID administration to the beginning of the active phase could improve recovery from a bone fracture. The three specific objectives are: (i) to critically analyze the available literature on animal studies on the effect of NSAIDs administration on bone healing; (ii) to estimate the extent to which the timing of NSAIDs administration and timing of surgery are associated with postoperative pain and bone healing in mice; (iii) to develop a protocol for a RCT to test the above hypothesis in a simple bone removal surgery in humans.

I address these objectives with three manuscripts: first, I conducted a systematic review and meta-analysis of animal studies on the effect of NSAIDs on bone healing after a bone fracture (Manuscript I). Second, I combined three experimental trials to estimate the effect of timing of

NSAIDs administration on pain and fracture bone healing; these experiments included pain behavioural tests of the mice with fracture surgery, bone healing assessments (micro computed tomography (μ CT), biomechanical bending, histomorphometric analysis), as well as the cytokines analysis and RNA microarray analysis (Manuscript II). Finally, I have developed a protocol, RCT, which is currently being conducted at the Jordan University of Science and Technology (Irbid, Jordan) (Manuscript III).

2 Literature review

2.1 Introduction

This literature review will provide an overview of the impact of bone fracture on individual's well-being and the financial burden for the society. Then, I describe the cellular and molecular events occurring during the bone healing process, particularly in the inflammatory phase. Subsequently, I will discuss the role of cyclooxygenases and NSAIDs on bone healing and describe the circadian rhythm process and its role in the inflammatory and immune responses. Finally, I will give an overview of the use of chronotherapy in the treatment of chronic diseases and its potential benefit in healing.

2.2 Epidemiology of bone fracture

The incidence of bone fracture is estimate to sturdily increase over the years (15), which will lead to noticeable financial burden and health wellness of the individual and the society. For example, it is estimated that bone fracture in the USA will increase from 2.1 million in 2005 to over 3 million fractures in 2025, solely based on the dramatic increase in the elderly population (16). The effects of bone fracture are devastating; in a given year in the USA, they can lead to 500,000 hospitalizations, over 800,000 emergency room encounters, 2.6 million physician office visits, 180,000 initializations (17), and over 100,000 non-union problems (3).

Failed and impaired bone healing occur in 5% to 10% of all patients who have suffered from a fracture (18). The treatment and productivity costs of uncomplicated fractures are of CAN$ 12,000 – 15,000 per case and, with the additional cost per case of delayed or non-union around CAN$ 12,000 (16). Moreover, there are several long term consequences of fractures including physical (e.g., physical impairment related to pain and functional), psychological(e.g., fatigue,

depression, anxiety and sleep disturbances), and social consequences (e.g., difficulty in returning to work and dependence on disability benefits) (19) (20).

Understanding the different biological and physiological phases of bone fracture healing will help to develop better treatment strategies to decrease the number of impaired bone healing and non-union.

2.3 Bone healing

Bone, the most abundant mineralized tissue in the body, is a type of connective tissue. It provides support to the body, protects soft organs, and helps in locomotion (21). It also serves as a reservoir for essential minerals (e.g., calcium, phosphorus) and functions as an endocrine organ (22). Bone has a very high rate of tissue remodeling (23). Bone can be divided according to the (i) shape into short, long, flat and irregular bones, (II) morphology into cortical and cancellous bone, and (iii) development to intramembranous and endochondral bone formation. Long bones such as tibia consist of metaphasis proximal ends and the bone body shaft called diaphysis.

Bone tissue consists of cells within extracellular matrix along with organic and inorganic components. The organic components of the matrix include collagen (primarily collagen I), and non collagen protein along with mucopolysaccharide. The inorganic components consist mainly of calcium and phosphorous in the form of calcium phosphate and carbonate (24).

Osteolinage bone cells are from different stem cells linage; while osteoblasts and chondroblast are from mesenchymal stem cells, osteoclasts are from haematopoietic cell linage (monocyte and macrophage linage) (25). Osteoblast cells, cuboidal in shape, are located near bone surface. They are responsible for bone formation by secreting matrix proteins such as collage type I protein and non collagen proteins such as osteopontine, sialoprotein and alkaline phosphate

(21). Osteocyte cells are terminally differentiated osteoblasts, which are able to sense mechanical loading on bone (23). These cells are important regulator of fracture repair and a key regulator of inorganic phosphate homeostasis. Osteoclasts cells are originated from haematopoietic origin stem cells which are differentiated to osteoclasts by two ligands or cytokines RANKL and macrophage colony-stimulating factor which released from osteoblasts cells which are responsible for the initiation of the osteoclastogenesis process for the osteoid tissue by the osteoclasts (26).

Bone healing can be categorized into direct and indirect patterns of repair based mainly on the amount of tissue loss along with the stability and proximity of the two fractured edges (27). Bone healing does not occur naturally by direct healing because it requires a correct anatomical reduction of fractured edges with a rigid compression and no gap formation. In this healing process, the cortex directly involves bridging the gap and produce the Haversian system through cutting cones which are osteoclasts tunnels into the bone matrix. Vascular endothelial and perivascular mesenchymal cells differentiate into osteoblasts which deposit extracellular matrix during the healing process, and there will be little or no periosteal response, therefore no cartilage formation (25).

Fractures in the diaphysis, where fracture gaps are larger than in metaphysis sites, usually heal via endochondral ossification which is healing by callus formation (24). This bone development process, which leads to the formation of soft callus for the stabilization of the fractured fragments when the fractured edges are separated, involves the intermediary tissue, the fracture periosteum, and the soft tissue. Undifferentiated mesenchymal cells undergo recruitment, proliferation, and differentiation into cartilage that is eventually calcified and replaced by bone.

These are the crucial steps in this type of bone healing, which involves hematoma, inflammation, angiogenesis, cartilage formation, cartilage calcification, cartilage removal, bone formation and bone remodelling (28).

2.3.1 Cellular and molecular signalling during bone healing

Researchers have investigated the temporospatial expression of cellular events and associated molecular ones in the healing of animals' bone fracture models. This evidence provides us with a comprehensive understanding of cellular events and some clues regarding the molecular signalling pathways involved in the process (24, 29-34).

During the initial stage of bone healing, which occurs within the first 24 hours after fracture and involves hematoma formation and inflammation, macrophage and other inflammatory cells as well as mesenchymal cells secrete pro-inflammatory cytokines and chemokines including interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), monocyte chemoattractant protein 1-MCP 1(CCL 2), monocyte chemoattractant protein 1α-MCP 1α (CCL3), platelets, bone morphogenic proteins (BMPs) (30). IL-1, IL-6, and TNF-α have a chemotactic effect on circulating inflammatory cells such as macrophages and play a role in the recruitment of mesenchymal cells (34). Using a closed, stabilized tibia mouse fracture model, Cho et al. showed that IL-1 and IL-6 reached peak expression one day after fracture and then decline to near undetectable level at day three, which concurs with the findings of other researchers (24, 34, 35). During the first three days, the platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) also play a role in bone repair (35). These growth factors induce migration, activation, proliferation, and angiogenesis of mesenchymal cells, alongside the chemotaxis of acute inflammatory cells (25). Mesenchymal stem cells proliferation and differentiation into a

chondrogenic or an osteogenic lineage will start from day three after fracture (33). From days 7 to 21, the chondroblasts proliferate into chondrocytes to form the soft callus that will help in the initial stabilization (34). The cartilage matrix of this soft callus includes type II collagen and proteoglycans. The hypertrophy and mineralization of this cartilage will subsequently occur in an organized manner (24).

Fibroblast growth factors (FGF), the vascular endothelial growth factor (VEGF) and angiopoietin 1 and 2 are essential to initiate the angiogenesis process at the early stage of healing. This process is characterized by vasculature invasion into the newly formed soft callus. The new blood vessels carry hemopoietic osteoclasts that start the resorption process. New woven bone formation, recruitment of mesenchymal stem cells and differentiating of osteoblasts at the fracture edges will follow at the end of this process (36). Angiopoietin 1 seems to be induced during the initial periods of fracture healing, whereas VEGF is induced later mainly during endochondral and bone formation (37). Osteoprogenitor cells contributing to the intramembranous bone formation come from sites away from the fracture. These cells originate from the cortical and periosteal tissue, and travel from the bone marrow site with high cellular density (30). The soft callus is gradually converted into hard callus by differentiated osteoblasts and committed osteoprogenitors from the cortex and periosteal cambium starting from day three. The growing woven or hard callus reaches its peak between day 7 and 10 and cease by day 14; however, the osteoblastic activity continues (24). The stimulation of undifferentiated mesenchymal stem cells and osteoprogenitors cells to differentiate into osteoblasts (osteoinduction) is a morphogenetic cascade involving discrete cellular transitions (35). The interaction between paracrine and

autocrine signals regulates this stimulation. Also, systemic hormones (e.g., parathyroid/thyroid, sex steroids), in addition to local factors and cytokines, may modulate these events (25).

2.3.2 Inflammation during bone healing

Inflammation appears to be crucial for both the initiation and outcomes of the bone healing process; in vivo experimental studies provide evidence regarding its role in the secretion of IL-1, IL-6, TNF-α, the macrophage colony-stimulating factor (M-CSF), and inducible nitric oxide synthase (iNOS) (Table 2-1). However, timing and termination of this inflammation are critical to the healing outcome; Serhan and Schmidt showed that the prolonged expression of pro-inflammatory mediators (e.g., IL-1, IL-6, TNF-α) is associated with delayed bone healing. When they compared the expression of anti-inflammatory cytokines and angiogenic factors (e.g., IL-13, IL-10, VEGF) in boney versus soft tissue hematoma, they observed in the former an elevated level and upregulated expression at 36h post trauma of stabilized sheep tibial bones (38, 39). Wu et al. 2013 analyzed data from several studies on the role of macrophages in bone repair and concluded that the tenacious polarization of macrophages to an M1 phenotype during the inflammatory and possibly early anabolic phase(s) might be unfavourable to fracture healing. They also emphasized that pro-inflammatory macrophages are short-lived; if these cells were present within the tissues outside the inflammatory phase, this could be associated with chronic inflammation and delay healing depending on the soft tissue injury, the duration and the microbial load after insult (40).

Recent molecular work on the role of macrophages, especially their polarization during bone healing, suggests that they can be the tip of the regulatory pyramid. Aspects of macrophage polarization investigated in several studies include the down- and upregulation processes behind

it, and its effect on the bone healing process (34, 41-43). Cross-talk between macrophages and mesenchymal cells seems to play a role in bone regeneration(38). Macrophages are of different phenotypes, and each one of them plays a significant role in bone repair. Interferon-gamma (IFNγ), lipopolysaccharide via toll-like receptors (TLRs) such as TLR-4, and the granulocyte-macrophage colony stimulating factor (GM-CSF) can polarize uncommitted (M0) macrophages to pro-inflammatory classically activated (M1) macrophages. On the other hand, the exposure of M0 or M1 macrophages to IL-4 and tumour growth factor-beta 1 (TGF-β1) can polarize them to an anti-inflammatory alternatively activated (M2) phenotype (44).

Scientists recognize the M1 macrophage phenotype by the increased expression of specific cytokines and chemokines such as TNFα, IL-6, IL-1, IL-12, Oncostatin M, and type 1 IFN with increased expression of iNOS, C-C chemokine receptor type-7 (CCR7), and HLA-DR antigen. Alternatively, M2 induces the secretion of IL-4, IL-10, IL-13, and IL-1ra and increases the expression of a cluster of differentiation (CD206), eosinophil chemotactic factor (Ym1), CD163, chemokine ligand 1 (CCL1), chemokine ligand 18 (CCL18), FIZZ1 gene (found in inflammatory zone), Arginase 1 (Arg-1), and chitotriosidase (45, 46). Macrophage polarization during bone healing seems to play an essential role in the healing outcome depending on the initiation environment and the phenotype expression through the pro- versus anti-inflammatory properties (28). The expression of different markers such as Ym1/2, Fizz1, and Arg-1 has been observed in association with the M2 polarization in mice models

More recent evidence, mainly from in vitro studies supported the links of M1 macrophage with bone destruction and M2 with tissue repair (47). More importantly, recent in vitro studies showed that monocytes and macrophages could promote the osteogenic differentiation of bone

marrow-derived mesenchymal stem cells, the precursor of the osteoblast (48). This process, which depends on prostaglandin E2 (PGE2) and cyclooxygenase 2 (COX-2), requires direct cell-cell contact leading to the production of a soluble factor. This soluble factor, identified as oncostatin M, induces a signal transducer and activator of transcription 3 phosphorylation (49). Tasso et al. 2013 demonstrated that mesenchymal stem cells in an inflammatory environment secrete a significant amount of PGE2, thus playing a vital role in macrophage polarization (50). Results from in vivo and cell culture studies demonstrated that cyclooxygenase inhibition with NSAIDs such as indomethacin could drive macrophage towards the inflammatory phenotype (51). The Goodman group conducted in vitro and in vivo experiments to investigate how macrophage polarization promotes osteogenesis by mesenchymal stem cells via COX and PGE2 pathways and how NSAIDs administration can affect bone healing. Lipopolysaccharide and TNF-α activate TLR4 and Tumor necrosis factor receptor 1 (TNFR1) on the migrated mesenchymal stem cells, which in turn, lead to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) signalling that increases COX-2 and PGE2 secretion. The PGE2 binds to EP2 and EP4 receptors on macrophage that increase the secretion of IL-10 and promote endochondral ossification. This process is also promoted through the induction of the COX-2 and PGE2 regulatory loop via STAT3 and the production of oncostatin M (52). The group also showed that the disruption of the COX-2 and PGE2 mechanisms by the administration of celecoxib leads to a dramatic reduction of bone remineralization. These results suggest that an inflammatory response via COX-2 and PGE2 pathways is essential for osteogenesis in contrast to the pathways of skeletal development (52). Also, the inflammatory environment mediated by macrophages negatively regulates

osteoprotegerin (OPG) secretion and may affect the osteoclast activity through the OPG- Nuclear Factor-kappa b Ligand (RANKL) axis.

Table 2-1: Phases during fracture healing in mice.
Temporal cellular and molecular signalling expression during fractured bone healing in mice (25, 34, 38, 53, 54)

Day	Event	Molecular signalling	
		Cells	**Signals/Cytokine**
1	**Hematoma formation and initiation of acute inflammation** (Activation and recruitment of proinflammatory cells and cytokines)	Macrophages, neutrophils and attraction of mesenchymal stem cells	***IL-1, IL-6, IL-8, IL-11, TNF-α, CCL2, CCL7, IP-10*** (from inflammatory cells- Monocytes /macrophage) ***PDGF, TFG-β*** (from degranulation platelets) ***BMP-2*** expression ***GDF-8*** and osteoprotegerin expression
3	**Fibrous tissue** (Angiogenesis)	Mesenchymal stem cells, osteoblast start to differentiate in intramembranous bone	Expression of: ***CCL3***, ***CCL11, IL-10, HIF1 α protein, HMOX, VEGF and GLUT1, TGF-β2, β3, GDF-10, BMP-5, -6 and RANKL and M-CSF, MIF (decreased for 2wks)*** Decline of cytokines: ***CCL7, IL-6, IL-1*** Induction of ***Angiopoietin-1***
7	**Soft and hard callus formation** (Endochondral ossification)	Intramembranous ossification's cell proliferation (peaks between 7-9 days). Maturation of chondrocytes (days 9-14)	Expression of ***CXCL12*** The peak of ***TGF- β2, β3 &*** osteoprotegerin Expression of ***GDF-5 and*** probably ***GDF-1***
14	**Cartilage resorption and Active osteogenesis**	Osteoblastic and osteoclastic cells activities, along with new mesenchymal cells	Decreased levels of expression for ***TGF-b2, GDF-5, and*** probably ***GDF-1*** Expression of ***BMP-3, -4, -7, and -8*** Expression of ***VEGF and RANKL, M-CSF*** Peak of ***IL-1 and TNF-α*** expression that continues during bone remodelling
21	**Remodelling**	Continuous osteoblastic and osteoclastic activities	Second peak of ***IL-1, IL-6, and TNF-α*** expression which continues during bone remodelling

2.3.3 Cyclooxygenase in bone healing

Two cyclooxygenase isoforms, COX-1 and COX-2, regulate the transformation of arachidonic acid (Aa) into final substrates through a specific prostanoid synthesis. The final prostaglandins are four substrates of Prostaglandin E2 (PGE2), prostaglandin F2α (PGF2α) and prostaglandin D2 (PGD2); prostacyclin (PGI2); and thromboxane A2 (TXA2) (55). COX-1 seems to regulate the function of many organs, while COX-2 is expressed mainly in response to trauma, for example, after fracture along with the initiation of the inflammatory process (11, 55-66). Experimental trials using COX-1 and COX-2 knockout animal models have confirmed the crucial role of COX-2 in maintaining bone density and strength, parathyroid hormone levels and other functions (67).

Prostaglandins are G protein-coupled receptors in the A-rhodopsin-like receptor superfamily. In bone tissues, the regulation of cell differentiation depends on the triggering of specific prostaglandins receptors which leads to the activation of different pathways (21). The binding of PGE2 to Prostaglandin E Receptor 2 and Prostaglandin E Receptor 4 activates the anabolic bone healing effect, while the PGE2 binding to Prostaglandin E Receptor 1 and Prostaglandin E Receptor 3 downregulates bone formation (68). The effect of Prostaglandin E Receptors on bone repair is different from one receptor to another. Zhang et al. using COX-1–/–, and COX-2–/– mice concluded that Prostaglandin E Receptor 1 expression inhibits bone repair (69), unlike when using an agonist for Prostaglandin E Receptor 2 and Prostaglandin E Receptor 4, which stimulate bone formation and promote endochondral bone repair (70).

O'Connor hypothesized that COX-2 has a critical function in chondrocyte differentiation during callus formation and endochondral ossification, a process that NSAID administration may disturb leading to delay or non-union healing. COX-derived lipid mediators appear to function through

the inflammatory phase and callus formation phase, with peak expression in the first three days and return to normal baseline level only after ten days post fracture (11). Besides the expression of pro-inflammatory cytokines (e.g., MCP-1, IL-6), which are essential for bone healing in the first three days, osteoprotegerin (OPG) appears to be expressed in high percentage in the callus at day 4 reaching a peak at day 7; this prevents premature resorption by the RANKL activated osteoclasts (59).

Lin and O'Connor (2016) reported the association of COX-2 expression with the osteoclasts callus and proliferating chondrocytes at day four after fracture, which suggests that the source of PGE2 synthesis after the initial inflammatory response comes at least in part from callus chondrocytes or osteoclasts that regulate fracture healing (59).

The physiological stress such as that from a fracture leads to an increase in the local activity of COX to produce PGs and promotes bone resorption and formation by the expressional influence of RANK and the reduction of OPG and RANKL. RANKL, which can be found on osteoblasts to activate the osteoclasts, is essential for bone metabolism and its action is promoted functionally by T-lymphocytes through the transcription factor NF-Kβ. As a counterpart of RANKL, osteoblasts also produce OPG, which is considered a protector of bone tissue as it acts as an antagonist by impeding the action of RANKL or RANK (71). Local hypoxia and other types of physiological stress in the bone tissue contribute to the synthesis of prostaglandins in the direction of growth and formation of osteoblasts and osteoclasts (72).

In conclusion, prostaglandins and COX-2 control critical phases of fracture bone healing, especially in the early inflammatory response of the endochondral ossification or bone formation

(55). Accordingly, evidence from in vitro and animal studies shows an association between the use of NSAIDs and delayed bone healing after a fracture.

2.3.4 Role of NSAID

Clinicians used NSAID frequently after a bone fracture or surgery due to their analgesic and anti-inflammatory effects (73, 74). However, using NSAID in an orthopedic context is controversial. More than 17 million Americans take NSAID daily, and up to 70 millions prescriptions are dispensed for these drugs each year (74). More than 25% of the prospective burden of drug interaction or side effects are related to NSAID (75). These drugs work by inhibiting both COX-1 and COX-2 enzymes, while selective NSAID inhibit only COX-2. From the mechanistics of bone healing, we can relate the effect of NSAIDS and those of inflammation and COX in the osteogenesis process after bone fracture. NSAIDs can negatively affect bone healing through different mechanisms that involve the immune system, mainly by inhibiting COX synthesis, which will affect the production of prostaglandin and other inflammatory proteins. As we saw previously, these processes appear to be essential for the regulation of bone hemostasis. Therefore, by inhibiting the production of PGE2 via COX, NSAID can have an adverse impact on the PGE2 in bone tissue, possibly causing a shift in precursor cell action towards bone resorption. Moreover, NSAIDs can disturb the equilibrium between RANKL and OPG in bone tissue, which in turn, may affect both processes in bone healing resorption and formation (21, 76).

Accordingly, animal studies have shown as early as 1975 (77) that NSAID administration negatively affects bone healing outcomes (11, 67, 71, 78); although evidence from fewer studies has revealed no effect of NSAID administration on bone healing (36, 79-82).

Studies have also investigated the mechanisms through which NSAID may delay bone healing. Evidence from animal experiments has shown that the blockage of COX enzymes through the administration of NSAID may lead to delayed or impaired bone healing(64). Moreover, Simone et al. revealed that COX-2 in comparison to COX-1 blockade is associated with impaired bone healing. Also, their work confirmed that administration of COX-2 affects the differentiation of mesenchymal stem cells into osteoblast cells during bone fracture healing. Several factors may influence the outcome of fracture bone healing in animal and may have impacted the results of these studies, including the age of the animal, type of NSAID and the duration of NSAID administration. In addition, variations on the time of NSAID administration during the day appears to lead to different healing outcomes; however, the relationship of this fluctuation with prostaglandins, COX and other pro and anti-inflammatory mediators variations during the day has not been investigated until now (61). Other factors that may affect the results of animal studies are the method of assessment and the time of outcome measurements. Studies that used histological assessment or bone mineral density (37, 80, 81) did not show the same deterioration in bone healing outcome as those using mechanical bending assessment (83-85).

Another line of evidence on the effect of NSAID in the healing process was provided by in vitro studies, which showed that all NSAIDs limit the proliferation of human osteoblasts by inhibiting the transition from the G0 to the G1 cell cycle phase (76).

Recent research has shown that introducing prostaglandins during the fracture healing process does not reverse impaired or delayed changes, which indicates that NSAID might affect bone healing through other mechanisms (21). These results coincide with those from Nagano et al. 2017 indicating that COX-2 blockade also inhibits the genes produced by the transcriptional

Wnt/-catenin canon signalling pathway, which plays a role in the proliferation and differentiation of osteoblasts precursors by blocking the osteoclastogenesis (86).

Clinically, results from many retrospective and prospective studies in humans have confirmed that NSAID therapy can significantly impair bone formation and bone healing following hip or femoral neck fracture, as well as hip arthroplasty (87-89) (18, 90). One study in humans has reported beneficial effects of NSAID on bone healing, particularly in Colle's fracture patients treated with casting and reduction (91).

These findings have even led some authors such as Marquez to suggest replacing NSAIDs' by opioids (92), taking into consideration the uncertain effect of opioids on healing and findings showing a relationship between high-risk non-union or reduced mineral density and opioid treatment. Such recommendations demonstrate that finding a new approach to use NSAIDs after bone fracture deserves more attention from researchers.

Considering the strong analgesic properties and fewer side effects of NSAID compared to other drugs after a bone fracture or bone surgery, there is an increasing interest to study the beneficial effect of NSAID on bone healing and regeneration. Indeed, a study by Jain et al. in 2014, has shown the beneficial effect of ibuprofen on preventing the bone resorption associated with the reduction of inflammatory cytokines (e.g., IL-1B and TNF-a) (93).

In summary, it is increasingly clear that the function of cyclooxygenases and their products are critical for bone healing, and most animal and human studies support the conclusion that NSAID administration, which inhibits cyclooxygenases, can delay or inhibit bone fracture healing. At the same time, it seems that the anti-inflammatory and analgesic effects of NSAID are very beneficial

in treating posttraumatic pain and edema. The need for a new approach to using NSAIDs that preserve its pain control properties without affecting bone healing is justifiable and important.

2.4 Bone fracture healing and pain

Bone fracture pain is a response to injury, which involves the induction of inflammation and the activation of nerve fibres (94). The aetiology of this nociceptive and neuropathic pain can be divided into four categories: i) mechanosensitive activation of bone sensory nerve fibres, ii) activation and sensitization of bone nociceptors due to inflammation, iii) ectopic nerve sprouting, and iv) central sensitization. Immediately following fracture, mechanosensitive nerve fibres in bone (C and A-delta nociceptors) are activated and rapidly transmit initial sharp fracture pain signals to the brain; increased intraosseous pressure due to bleeding and edema can also trigger pain signalling. Subsequently, bone fracture induces inflammation, ectopic nerve sprouting and pathological hyper-innervation of sympathetic and sensory nerve fibres into the bone (marrow, mineralized bone, and periosteum). Within minutes to hours of the fracture, cells at the fracture site release cytokines and growth factors, such as Nerve Growth Factor, which acts on tyrosine receptor kinase A and sensitizes nerve fibres in the bone (95, 96). This results in local mechanical hyperalgesia, and localized tenderness during the bone healing process, such that normally innocuous stimulation are perceived as noxious (97, 98). This process, which is important for regeneration at the injured site, drives a dull aching pain at rest and sharp pain upon movement, and if rapid and effective healing of the fracture does not occur, it could develop into chronic bone pain (95).

Bone fracture healing and pain are influenced by many phenomena including the bone healing process and the circadian rhythm (99). The relationships between these phenomena and pain

are not well understood but could be critical for the development of better strategies to manage bone fracture pain while avoiding interference with bone healing (99). Bone fracture healing follows four sequential and overlapping stages (homeostasis, inflammation, proliferation and remodelling), which are intertwined with the circadian rhythm.

2.5 The circadian rhythm in immunity, inflammation, bone metabolism and healing

2.5.1 Circadian rhythm in immunity and inflammation

The term circadian rhythm comes from the Greek word “circa,” which means “about the day.” It was introduced by Halberg to describe endogenous oscillations in organisms that were observed in close association with the earth's daily cycle (13). In mammals, many biological functions are modulated by the circadian timing system as a result of different endogenous oscillations at the genetics, cellular and metabolic levels influenced by external cues during the 24-hr cycle (100). The circadian system in mammals is composed of a central clock at the suprachiasmatic nuclei (SCN) and a peripheral clock inside all cells. The SCN controls the rhythmic regulation of physiological processes, involving the hormonal and autonomic nervous systems and temperature. Also, the SCN contributes to the regulation of autonomous molecular clocks located in each nucleotide cell, which then oscillates the expression of a considerable number of genes involves in intercellular processes.

A feedback loop of heterodimer core clock genes, specifically circadian locomotor output cycles kaput (Clock) and brain and muscle Arnt like protein-1 (Bmal1), controls the circadian clock. Clock and Bmal1 drive the expression of two inhibitors, cryptochrome (Cry) and period (Per)1, 2 (101). The core clock proteins Bmal1, Clock, and nuclear receptor Rev-Erbα, and their repressors

proteins Per and Cry control fundamental aspects of the immune response and bone healing process (101).

Circadian regulation does not only coordinate the clock core mechanism, but modulates many downstream processes (102). More than 40% of the transcriptomes is believed to oscillate in circadian rhythm along with 20-40% of proteome and metabolites (103).

Thus, clocks cause circadian oscillation in the activity of immune cells and their associated cytokines in innate and adaptive immunity in response to external insults and inflammation, both at the cellular and molecular levels (13, 100, 104). Macrophage activity, leukocyte recruitment, and pro-inflammatory mediators such as interleukin-1β (IL-1β), IL-6, and IL-12 are more pronounced at the beginning of the daily activity phase. Moreover, there is an increased activity of Tol-Like Receptors (TLR) TLR9 and TLR4 that upregulate the expression of CCL2, CXCL1, and CCL5 leading to activation of leukocyte recruitment and potential tissue damage at the injured site (100, 101, 104, 105). By contrast, regulatory mediators such as the vascular endothelial growth factor (VEGF) peak during the resting phase (101, 106, 107) (Table 2-2).

Recent studies suggest that the cyclic rhythm in recruiting immune cells to the tissue can affect the expression of diseases and the effect of therapeutic approaches. The clinical importance of the circadian expression of innate immune system components on the onset and exacerbation of inflammatory diseases (e.g., rheumatoid arthritis, periodontal diseases) may be a crucial factor in the healing process of hard and soft tissues (13, 101-103, 108-110).

Table 2-2: Different immune cells and cytokines circadian rhythm expression during the day in human and mice.

Cytokines/ Chemokines ligands or receptors/	Role in inflammation and bone healing	Circadian Rhythm	Reference
IL-4	Th2 cytokines that are associated with macrophage M1-M2 polarization. Expresses at 3-4 days after fracture associated with anti-inflammatory macrophage trait	H-ZT18	(111) (53)
VEGF	Secreted by endothelial cells, induces angiogenesis and growth. Expresses at the end of hematoma formation when the hypoxia state starts (around 4 to 14 days after fracture)	M-serum Peak during the dark phase and lower levels at light phase (ZT2 and ZT14)	(107)
IL-6	Cytokine largely responsible for inducing the synthesis of the acute phase proteins, C reactive protein and serum amyloid protein A (SAA), and is one of the major cytokines involved in bone resorption. Highly expressed on day 1 and with the presence of inflammation expressed at day 7 and 14	H-Morning 7:30 Healthy and RA patients at 4:00 am	(112), (113) (34)
IL-10	Healing in the fetus (114), expressed at day 4-7 after fracture	H-Morning 7:30 with the second peak at 13:30	(112)
TNF-a and its receptors (p55 and p66)	Expressed at day 1 and later at day 21 and 28, depressed level at day 7	H-S-Morning 7:30 6:00 am in healthy and RA patients	(112) (34)
MMP-9	Potent degenerative enzyme	H- Increase 200-fold on awakening	(115)
IL-1B	One of the most potent osteoclast-activating factors within the human organism and thus believed to play an important role in periodontal tissue breakdown, IL-1 peak on the 3rd day after fracture, expressed at day 1 and later at days 21 and 28, depressed level at day 7	H-GCF-Periodontal healthy subjects-lowest morning, highest evening (melatonin can affect)	(116) (30)
Osteoprogetron (OPG) and its receptor (RNKL)	Peaks in the fracture site after 24hours and at the peak of cartilage formation phase at day 7, while RANKL osteoprotegerin was seen on day 3 and 14 like MCSF		(34)
IL-1R1 and receptors	Expressed on day 1 and 3 post fracture		(34)
IL-5	Recruitment and activation of the OPG and inhibition of osteoclast activity		(117)
Macrophage inhibitory factor (MIF)	Expressed at day 4 of fracture and decreased gradually for two weeks. Counter-regulator of glucocorticoid action.	H- peaks 6:00-9:00 am Nadir: 00:00 to 3:00	(118), (119)

	Induces immunosuppression and glucocorticoid Induces proinflammatory cytokine inhibition 8% of macrophage cells and their secretion is under local circadian control-IL-6 or TNF-a		(120) (104)
CXC chemokines ligands CXCL12	Induced recruitment and retention of hemopoietic stem cells, macrophage cells and mature immune cells, Upregulated during the acute phase of bone healing	Downregulate at the beginning of the resting phase to allow the release of the cells in the blood (ZT23, ZT1, ZT24)	(53)
CXCR4	The receptor of the CCL12 on the cells and CD4, CD8 and T cells. Associated with the recruitment of BM-MSCs to the fracture site	Peak during the resting phase	(53)
P-selectin, E-selectin, VCAM-1 ICAM-1, Ccl2	Initiation of the inflammatory phase of the endochondral ossification healing. Express on day 1 – day3	M-bone marrow –(ZT13)	(53)
INF	Role in initiation and activation of macrophage polarization from M0 to M1. Expressed on day 3	Natural killer -M – (ZT 14-24)	(53)
TLR9 Toll-like receptor 9	Modulates the inflammatory response during bone healing and may affect the osteoclastogenesis through its effect on osteoclasts cells or osteoblasts. Subunit of TLR family	M-ZT19-7	(13)
Neutrophils, lymphocyte, monocyte, eosinophils	Recruited to the fracture site for secretion and initiation of the inflammatory phase Highly expressed at day 1	M-CT17 (during active phase)	(43)
Macrophages	Recruited at the beginning of the inflammatory phase at the fracture site (role in the secretion of different proinflammatory and anti-inflammatory cytokines)	M-CT0-12 (during resting phase)	(104)
B-cells and T-cells	During the later stage of inflammatory phase, B-cells and T-cells are involved in suppression of the pro-inflammatory signals, and able to induce anti-inflammatory functions from mesenchymal stem cells and induces osteogenic differentiation and activity	M-ZT5-13	(121)

H-Human, M-mouse, CT: actual circadian time in hours (e.g. CT6 = 6 AM); ZT: Zeitgeber time: time after the onset of light with lights on at ZT0/24 and off at ZT12, ZT 0-light on, ZT12-Light off; S-serum.

2.5.2 Circadian rhythm in bone metabolism and healing

Evidence for the role of the circadian rhythm in bone metabolism and healing was collected at different levels: in a single cell, a population of cells, laboratory animals or human subjects.

Collectively, these studies have shown that the transcription of half of all protein-coding mammalian genes is affected by the circadian rhythm in at least one tissue. In other words, they show clock control, which is transmitted to their proteins and even metabolites (108, 122, 123). All bone cells (e.g., osteoblasts, osteoclasts, and chondrocytes) express clock genes such as Per or Cry that influence bone volume regulation (124, 125). Cry2 mainly influences osteoclastic activity, whereas Per2 regulates osteoblastic activity (126). Accordingly, bone and cartilage metabolism is affected by the circadian rhythm (127), with more bone formation occurring during the resting period and more resorption during the activity period (128). Experimental studies in rodents and humans also reveal that sleep and circadian disruption impair bone formation (129). The 24-hour oscillation has been observed in bone tissue during growth (130), formation, resorption (128, 131), and even in endochondral ossification during fracture bone healing (128).

Early histological and biochemical studies, such as the work of Simon et al. in the late 60s to the 80s, demonstrated that bone metabolism is affected by the circadian rhythm. However, the authors had difficulty understanding the underlying molecular mechanism (64, 65). Studies in rabbits have shown a 24-hour variation in the long bones growth process with the highest bone growth rate during the resting phase; endochondral ossification and epiphyseal cartilage mineralization also show a circadian rhythm (66). Their findings concur with Greenspan et al. who demonstrated that bone resorption markers (e.g., carboxy-terminal cross-linked telopeptide of type 1 collagen that is generated by cathepsin K activity) have a circadian rhythm with its highest serum concentration at the beginning of the active hours of the day. Also, bone formation markers (e.g., osteocalcin and alkaline phosphatase (ALP) have their peaks at the beginning of the resting time of the day (67, 68).

Kunimoto et al. 2016 showed that endochondral ossification healing in a mouse femur fracture model involves circadian clock genes and transcripts (71). Moreover, clinical and retrospective studies proved that timing of skin wound or cardiovascular surgery could affect the healing outcome and post-operative recovery (71, 72).

The immune response after injury, which is carefully controlled by circadian clocks, is crucial to control the pathogenic and host healing process (103). The circadian clock controls the timing and duration of acute inflammation to confine the pathological challenges, and timely start the healing process without going into chronic inflammation, which will delay healing. (110). However, a comprehensive understanding of the inflammatory mechanism linking this oscillation to the precise molecular level has yet to be achieved.

2.5.2.1 The circadian clock could be the key

The 2017 Nobel prize for medicine was given to three scientists for their discovery of the intrinsic rhythm generated by endogenous circadian clocks (132). Indeed, the circadian transcriptome analysis of multiple organs/ tissues from the same animals has revealed that nearly all genes in the genome show circadian modulation (102). Circadian clock transcripts modulate the circadian rhythmicity of different hemostatic functions in multiple tissues involved in immunity, cellular defence and communication, and other functions (110). Two approaches can be identified from the literature regarding the implication of the circadian rhythm and clock for health and disease control, including drug administration.

- Chronotherapy (timing the drugs)

Chronotherapy is the science of preventing or treating illness according to biological rhythms (133). It involves the timing of pharmacological and non-pharmacological interventions such as

surgery, activity and other therapy to minimize side effects and increase treatment efficacy. It also aims to target treatments or drug administration for better healing outcomes depending on the biological circadian rhythm of the healing processes (134-136).

Preclinical experiments in the 1960 and 1970s demonstrated reproducible increases in toxicity as a function of circadian timing over fixed timing doses for several drugs like methopyrapone (an adrenal cortical inhibitor), morphine, lidocaine hydrochloride, oubian (an antihypertensive drug), methadone, arabinosylcytosine, cyclosporine, and lithium (137). Although the potential implications for reducing adverse events of treatments were already emphasized, the results generated scientific controversies, which usually originated from methodological issues regarding animal characteristics, synchronization, and manipulations (134). These studies resulted in the demonstration of circadian dependent pharmacology for more than 400 drugs, including NSAID, which lead to their chronotherapy application in mice or rats through different routes (137).

There are 24-hr changes in the process that determines drug's pharmacological characteristics, including absorption, distribution, metabolism, excretion and elimination. The 24-hr changes in the drug chronopharmacokinetic are related to physiological circadian changes that involve the gastric PH, plasma protein and protein subtypes, and they can be affected by changing or imposing a feeding routine (138).

Chronopharmacodynamic refers to the drug activity that is modulated by the circadian rhythm of its direct intercellular target and triggered pathways, and also the extracellular environment circadian status as a result of control by the circadian timing system of immune and inflammation and other physiological functions (137). Levi et al. harvested bone marrow cells at different time

points and found that the topoisomerase II inhibitor toxicity toward these cells depends on the time of collection (108). This approach can be used to test implications for different physiological functions.

Overall, circadian gene expression results from rodents and human tissues indicated that more than 80 % of FDA approved drugs have a circadian rhythm in mRNA levels and many of them also may have a daily rhythm in their particular functions (139). Successful chronotherapeutic results have been demonstrated in treating inflammatory diseases such as rheumatoid arthritis, hypertension and even in cancer therapy (140). Despite these encouraging outcomes from the chronotherapy approach, there is still a need to consider additional factors to ensure the maximum benefit and personalized benefit from this approach. These factors may include the correct target, appropriate drug, and the appropriate dosing. Also, it is imperative to highlight whether the daily rhythm in a disease or injury condition is the same as in the normal condition; if it is the same, it will be very wise to target the peak time of the pro-inflammatory response.

- Administration of drugs can affect the circadian clock

There is a rising interest in targeting the circadian clock as a therapeutic approach to control diseases or promote healing (102). As the circadian clocks are coordinated, several cellular pathways and clock components will maintain the hemostasis by transiently repressing a pathway, thereby avoiding chronic activation. For example, after injury, the circadian rhythm represses inflammation and prevents it from progressing toward the chronic state. Targeting the clock components can suppress the inflammatory response at the right time to restore the normal circadian rhythm of the healing process will be most valuable. Interestingly, as many circadian clocks have molecule ligands that potentiate their function, several drugs-like

compounds have been developed to target these clock components and have shown beneficial results in the in vivo preclinical studies. Most of these studies focused on inflammatory diseases, metabolic diseases, and cancer (141).

2.5.3 Circadian rhythm and NSAID administration

Chronotherapy is a therapeutic strategy whereby the timing of drug delivery is based on the circadian rhythm of physiological and pathological activities, and it aims at synchronizing drug activity with this variation (107).

Part of the pain response oscillation could be explained by COX-1 and COX-2 significant circadian changes during the day (142), especially after an injury or insult (143). This is probably why laboratory and clinical evidence show that there are circadian and circannual variations in the pharmacokinetics effects of NSAIDs. These findings suggest that maximum absorption and effectiveness are achieved when the drug is administered during the active phase (110, 134-136, 144).

The circadian response of human individuals gives rise to substantial differences during the 24-hr cycle in their risk of severe medical events (e.g., cardiovascular accidents) and symptoms of chronic inflammatory conditions (e.g., allergic rhinitis, asthma, rheumatoid arthritis and osteoarthritis). The same prominent circadian time structure can substantially affect the kinetics and dynamics of many classes of medications when used at different biological times of the day and night. The results obtained in different clinical studies strongly suggest that careful selection of administration time can improve the effectiveness of NSAID and markedly reduce their undesirable effects. They exert a strong anti-inflammatory effect when ingested or injected in the morning or early afternoon, but not in the evening (138).

Understanding the circadian biology, especially immunity and inflammation after injury or insult, may provide the information needed to optimize the timing of NSAID administration to maximize its efficacy and minimize side effects such as indigestion, stomach ulcers, and acute kidney problems (145).

3 Statement of the problem

Bone fractures are extremely painful and can cause significant morbidity and even mortality in some cases (4). NSAIDs' are the most common drugs used following the treatment of bone-related surgeries. However, animal experiments (31, 146-149) and human studies (18) indicate that the administration of NSAID may impair the healing of fractured bone (11, 37, 77, 85, 146-148, 150-163) due to their impact on the inflammatory process.

The immune response involved in the inflammatory process, including those after fracture or injury follows a circadian rhythm (13, 100). Indeed, clock gene expression has been proved in different bone cells that affect bone hemostasis and bone growth during the normal physiological process or bone fracture (128, 164, 165). Moreover, there is a proof of concept that skin healing differs depending on the time of the day when the injury occurred (166). Also, the circadian biology may affect healing outcomes of surgical procedures in different parts of the body (167).

In addition, the timing of medication taking into account the circadian rhythm, also known as chronotherapy, seems to have an effect on the inflammatory process. This has led to recommendations of different administration schedules for many inflammatory diseases and conditions including chemotherapy regimes for cancer treatment. The latter seems to improve patients' quality of life.

The effect of NSAID seems to exhibit a daily rhythm (139). However, we are unaware of any studies investigating the effect of NSAID chronotherapy on post-surgical pain and bone healing. Moreover, the studies have not reported surgery time. This is a major issue since the inflammatory response follows the circadian rhythm.

The overall aim of my PhD project is to further our understanding of NSAID use in the healing of bone fractures. To achieve this, I propose an interdisciplinary project that encompasses the entire spectrum of scientific inquiry. My first paper is a systematic review and meta-analysis of preclinical trials on the use of NSAID after bone fracture surgery. The review integrated a quality assessment of the retained studies and adopted the risk bias tool and methodology specified for animal studies. Following this review, I conducted a series of pre-clinical animal trials, which tested the chronotherapy hypothesis. One of these trials included the identification of the systemic cytokines and gene expression markers of different biological processes occurring during the healing period of the bone fracture surgery after the administration of NSAID at different times of the day. Finally, I developed a protocol for a RCT to test the NSAIDs chronotherapy approach among humans.

4 Aim and objectives

The overall aim of the project is to better understand the effect of NSAIDs chronotherapy on bone healing after fracture surgery. The working hypothesis is that the administration of NSAID during the active phase after bone fracture surgery results in better recovery and bone healing in comparison to administration during the resting phase. The specific objectives are:

i) To conduct a systematic review and meta-analysis to estimate the effect of NSAID administration on bone healing biomechanical and histomorphometric measurements in different animal models after bone fracture surgery.

ii) To estimate the extent to which NSAID administration during the active phase is associated with lower expression of pain behaviour and better healing outcomes in comparison to NSAIDs administration during the resting phase, using a tibia fracture model in mice.

iii) To identify the inflammatory cytokines and metabolic pathways affected by NSAIDs administration during the active phase compared to NSAID administration during the resting phase in a tibia fracture model in mice.

iv) To prepare a RCT protocol to estimate the extent to which the administration of NSAID in the morning and early afternoon, compared to three doses during the day, is associated with a decrease in pain and postoperative recovery after third molar surgical extraction in subjects attending the dental clinics at the Jordan University of Science and Technology

5 Methodology

The three manuscripts of this thesis include a systematic review with meta-analysis, a manuscript presenting the results of a series of experimental trials and the protocol of a RCT. This chapter details the techniques I performed in this thesis for each manuscript. Subsequently, the methodology of the studies involved in each manuscript is explained in detail, and also synthesized in each of the manuscripts.

5.1 Systematic review and meta-analysis in animal studies

A literature review that includes a comprehensive method of summarization all the available relevant studies and may include synthesization of evidence regarding a specific question by conducting a meta-analysis is referred to as a systematic review (168).

The systematic review and meta-analysis of animal studies are relatively novel. It is documented that the first systematic review of animal studies was conducted in 2001 by Horn et al. (169). The number of published systematic reviews of animal studies is rapidly increasing after 2001. Cochrane Collaboration supported centers that are promoting Cochrane similar approaches for conducting systematic reviews of animal studies; the first one is the Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES; www.camarades.info), which was established in 2004. Another centre is the Systematic Review Center for Laboratory animal Experiments (SYRCLE; www.umcn.nl) research group, which is providing training courses to help the researchers on how to conduct a systematic review of preclinical studies and use the results to develop and improve methodologies and guidelines.

Data from the systematic review of the animal studies can help to reduce the risk of bias by outlining the clarity of aims, objectives and methodologies. They also highlight gaps in the literature and help to improve the design of future experiments by avoiding unnecessary replication and providing evidence-based input in choosing the animal species, a specific dose of medication, or what outcome to measure. Moreover, it is important to emphasize on the exploratory nature of this review mainly on sources of heterogeneity and not to determine how "good" a drug is or to decide about the safety of health care approach (170).

Same as in the systematic review of the clinical trials; there are several tools have been developed and validated to help in the search (168, 171), and the meta-analysis approach or for the risk of bias assessment (170, 172).

The phases to follow in conducting a systematic review of animal studies start with the formulation of the research question and identification of the inclusion and exclusion criteria. After that a screening and reading of the abstracts and full-text papers to identify the included studies, followed by extraction of the required data and evaluation risk of bias and the quality of the included studies. Conducting meta-analysis depends on the availability of the data (173).

A meta-analysis of the included animal studies will be conducted if it is applicable. One of the main objectives of animal studies meta-analysis is to explore heterogeneity among the studies. Then choosing the effect size measure of specific outcome followed by estimation of the effect size either by random or fixed effects model. The subgroup analysis can be performed if enough data is available, and there are enough group comparisons to estimate a difference. A meta-analysis of the animal studies may include sensitivity analysis depending on studies quality assessment. Also, publication bias may be performed (173)

5.1.1 Methodology used in the first manuscript

The Cochrane methodology used was specified and documented in advance using the SYRCLE systematic review protocol for animal studies (Appendix I-Table 12-1S) (168). The protocol was published in the SYRCLE database.

5.1.1.1 Amendments to the review protocol

I did not assess the effect of low-quality studies through sensitivity analysis because of low scores among studies for at least one of the domains used to determine their quality.

5.1.1.2 Search strategy and selection of studies

We searched eight databases (Embase, Scopus, Medline, CINAHL, BIOSIS, Cochrane, Central, and DARE) for original articles concerning the effects of NSAID on fractured bone healing in animal models from the inception of each database to August 1st, 2017. The search strategy was developed with Martin Morris, the Dentistry librarian from the Schulich Library, McGill University, Montreal, Canada. We did not have a lower limit for the search strategy. The strategy was composed of three elements: anti-inflammatory agents/non-steroidal, bone, and animals (for the complete search strategy, see Appendix I- Table 12-2S). We drafted and used a search filter to detect all animal studies. Additionally, we screened the lists by hand to check for relevant articles. No language or other restrictions were used, other than excluding human studies.

Two independent reviewers (Haider Al-Waeli, Ana Paula Reboucas) screened the selected papers using RAYYAN software (httpp://rayyan.org, Doha, State of Qatar) (174), and any disagreement was solved by discussion, or by including a third reviewer. Full-text articles screened were included when they met pre-specified criteria: (i) a controlled NSAID interventional design after bone fracture; (ii) description of outcome measures related to bone healing (biomechanical

characteristics, μ-CT scan measures, radiographic bone assessment, histomorphometric and histological based grading). We excluded papers that fulfilled one the following criteria: (i) not an original study (e.g., reviews, letters); (ii) use of a bone graft or other materials; (iii) measuring only outcomes that were not biomechanical or histomorphometric; and (iv) duplicates. The registered protocol contains these inclusion and exclusion criteria in advance (Appendix I-Table 12-1S).

5.1.1.3 Data extraction

In addition to authors and year of publication, we extracted information from selected articles related to the animal model used, weight and sex of the animals, type of fracture model and bone, whether there was fixation or not; type of fixation method; number of fractured bones, fracture site on the bone, use of opioids and antibiotics, NSAID intervention type, name, duration and route of administration, outcome measure and time point of data collection, and the reason for exclusion of a study from the meta-analysis. Outcome measures related to bone healing were divided into biomechanical characterization (maximum or ultimate force load (MF), stiffness (Stiff.) and work to failure (WF)); histomorphometric characterization (any quantitative study on μ-CT images (bone volume (BV), bone volume to tissue volume (BV/TV), or microscopic image of fracture callus (bone percentage or mineralized tissue percentage (B%)) (Table 5-1).

We extracted all the available data, including experimental groups means or medians, standard deviation (SD), standard error (SE), number of animals per group (n) for all continuous outcome measures. Attempts were made to obtain original data by contacting authors if results were presented by graphs or incomplete only. If data could not be retrieved, the study was excluded from further analysis.

Table 5-1: Study characteristics

Study information	
	Author name(s)
	Study name
	Journal name
	Year of publication
	Location
Population	
	Animal species
	Age of animal
	Sex of animal
	Weight of animal
	Strain of animal
Intervention (fracture and medication)	
	Fracture type
	Technique used to perform the fracture
	Type of fractured bone
	Number of fractured bones
	Site of the fracture on the bone
	Type of fixture after bone fracture
	Type of NSAID
	Name of NSAID
	Dose of NSAID
	Route of administration of NSAID
	Interval and duration of NSAID administration
	Use of antibiotics
	Use of opioid
	Use of another analgesic
Inclusion and exclusion	
	Is the study included in the meta-analysis
	Reason for exclusion from the meta-analysis
Study outcomes	
	Type of biomechanical bending
	Time to measure biomechanical bending after bone fracture
	Type of other outcome measurements
	Time to measure other outcome measurements

5.1.1.4 Risk of bias assessment

Risk of bias assessment tool used to assess the quality of the included studies. The assessment was done by myself and another reviewer (Alaa Mansour) using SYRCLE's risk of bias tool (172) This tool is based on the Cochrane Collaboration tool for assessing the risk of bias, and it was adjusted to assess bias in animal studies. The tool helps to assess six types of bias (selection,

performance, detection, attrition, reporting and other) distributed into ten domains. If the answer to the related domain was yes this indicates a low risk of bias, if the answer was (no) it indicates a high risk of bias, and the third score was (?) if the bias was not clear.

To overcome the problem of recording many items as unclear risk of bias, we added four domains (175): reporting any measure of randomization, mentioning any blinding measure, describing sample size calculation, specifying the time of the day at which NSAID was administered or the time of day at which surgery was performed (172). The scores were either (yes) if reported and (no) for unreported items.

5.1.1.5 Data analysis

We applied the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines to conduct the meta-analysis (176). We used Comprehensive Meta-Analysis software (version 2.2.064, Biostat Inc., Englewood) when five or more independent comparisons from at least three different studies for each outcome were included (if these assessments were sufficiently comparable for each outcome). We calculated the standardized mean differences (SMD) through Hedges g effect sizes (177). The calculation was (SMD = the mean of the NSAIDs group minus the mean of the control vehicle group divided by the pooled standard deviations of the two groups) to account for the differences in measurement units. Hedge's g is based on Cohen's d, used to correct bias due to the small sample size (170).

The individual effect sizes were pooled to have the overall SMD and 95% confidence intervals. We used a random-effects model, which takes into account the precision of individual studies and variation between the studies, and weights each study accordingly (173). Heterogeneity was

expected among animal studies, and it was estimated by the proportion of total variance and reported as I^2. Moreover, we calculated the between-study variance.

If the study includes one control group that is compared to several independent experimental groups, then the number of the animal in the control group was adjusted by dividing it by the number of the included experimental groups.

For different healing outcomes measurements, we assessed the publication bias by examined funnel plot asymmetry if the analysis contained at least 20 comparisons.

Subgroup analyses were conducted to explore possible sources of heterogeneity and to assess which variables influence the effect of NSAID on bone healing outcomes. The results were interpreted if the subgroup included at least ten comparisons. A minimum of three independent comparisons per subgroup was needed to record the subgroup characteristics, which was followed by subgroup analysis, as prespecified in the registered protocol. Subgroup analyses were considered for animal species (mice, rats, rabbits), sex, type of NSAID (non-selective, Cox-2 selective), type of fracture, and period of outcome measurement or data collection (early healing less than 21 days, 21 to 48 days and more than 48 days).

5.2 Techniques used in the preclinical experiments

5.2.1 Pain behaviour assessments

Pain in animals, including mice, cannot be measured directly. Therefore it was essential to develop methods for pain measurements through animal behaviour or nociception (178). The pain response is generated by activation of the nociceptive nerve fibre through external stimuli such as heat, which elicits an electrical signal that will transmit later to the spinal cord and then

the brain which leads to a withdrawal response (179). It is difficult to clinically translate evoked pain response due to external stimuli, such as mechanical von Frey, Randall-Selitto and Hargreaves tests. Therefore, there was a need to develop and implement a non-stimulus pain evoked methods such as weight-bearing and guarding analyses (178).

5.2.2 Weight-bearing and guarding test

Static weight bearing or incapacitance tests measure weight distribution of the hind paws in an inclined holder which is forcing both hinds' positions on two independent pressure sensors. After surgery or treatment in one paw, there will be a different distribution of the weight between the injured leg and the contralateral one. The difference in weight distribution is considered as the body adjustment to the nociception response. The differences in weight distribution between the two hind legs have been noticed and used in models other than surgery such as osteoarthritis, bone cancer-induced pain, carrageenan-induced inflammation and sciatic nerve crush injury (180). It takes time to get the animal into the correct position to measure the weight distribution because, during the test, we allow the animal to adapt freely without any restraint. Unlike dynamic weight bearing or guarding analysis; static weight bearing can only be used to assess the nociception response in a model that includes treatment or surgery of unilateral hind paw or leg. In addition, the static weight bearing test needs extensive training to the animal and the investigator (181).

Gait analysis through guarding test is widely used to estimate the non-stimulus or spontaneous pain response in animal studies. The guarding test allows interpretation of spontaneous nociception pain response in both hind paws, and the interpretation depends on the gait analysis. Gait analysis methods may include measuring and analyzing different parameters such as, paw

pressure and weight distribution (paw intensity), or paw printed area, length and toe spread, or parameters from the dynamic animal movement such as swing phase or speed, and interlimb coordination (181). The concept of the guarding test depends on the animals' response to spontaneous pain; after surgery the animal will guard the painful paw and changes its gait and move with a limp or changes the stride size. This can lead to a different score or grade depending on the different intensity of the paw pressure (182). The intensity or the pressure of the guarding paw can be either visualized live through direct monitoring or after video recording; the paw pressure intensity can be scanned or recorded digitally through different methods that could include light or colouring ink (183).

5.2.3 Microcomputed tomography (μ-CT)

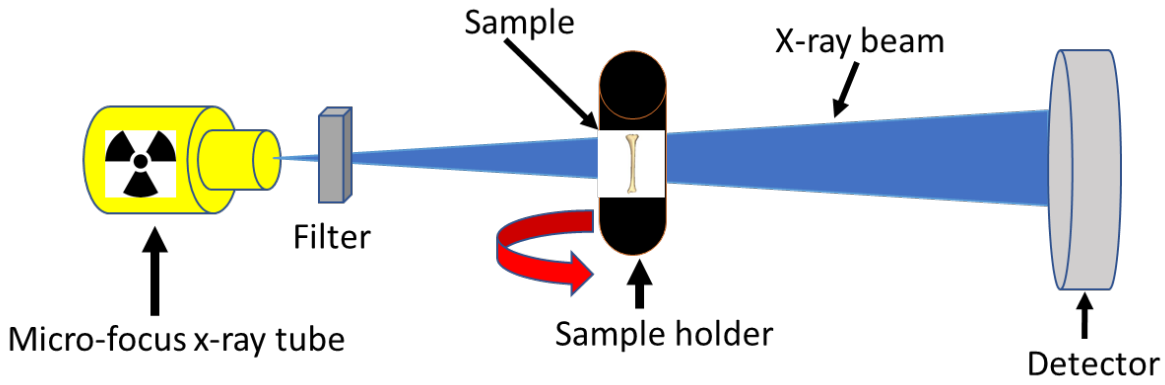
Microcomputed tomography is a procedure that generates three-dimensional images of an object's external and internal structure without sectioning of the specimen. An important advantage of this technique is that it allows the object to remain intact for further analysis. The use of high-resolution μ-CT imaging to investigate mineralized tissues, soft tissues, and biomaterials has grown immensely in recent years (184). One of the reasons for the increased use of the μ-CT in the research because it can deliver accurate quantitative and qualitative data regarding the morphology of an object. Compared to conventional histological 2D imaging, μ CT analysis can provide faster throughput measurements of significantly larger volumes of interest. Another advantage of using μ-CT is that it can be used to provide images from both live animals (in vivo scanning) or from extracted specimens (ex vivo scanning) (185).

μ-CT systems can be used to scan different materials (e.g. Scano, SkyScan, XRadia), by adjusting the scanning parameters (184). To obtain precise and reproducible information from a μ-CT scan,

it is imperative to establish standardized clear protocol that can be replicated on different specimens by different users (185). In general, μ-CT analysis is a reconstruction of a 3D image form two dimensional x-rays images of a specific sample by using computer algorithms (185, 186). These images or projections are created by setting the x-ray source and the detector in a fixed position while the sample object rotates around its axis (Figure 5-1).

The source of the x-ray is usually a micro-focus x-ray tube, and the generated x-ray is converted into visible light by a detector that is composed of a charge-coupled device camera with a phosphor layer (184, 186). Upon irradiation of an object, some of the x-rays are attenuated while they across the object and others are captured by the detector and depending on the difference in radio density. A two-dimensional images are created for the object reveal the different morphological structure of the object (e.g. cortical bone and cancellous bone within a bone sample, or empty pores within a scaffold matrix) depending on the particular attenuation coefficient which is corelate with the certain material density (184-186). Computer three-dimensional modelling software, such as SkyScan CT-analyzer, stacks the two-dimensional maps to generate three-dimensional models and perform various analyses. It is crucial to select appropriate software and hardware facilities to perform the radiographical assessment effectively and efficiently (186). In summary, the μ-CT procedures consist of four general steps: (i) sample preparation (pre-scanning); (ii) obtaining the x-ray projection images (scanning); (iii) reconstruction of the projection images into 3D images using computational models; and (iv) analysis of the 3D image stack using computer software, in which several outcomes can be evaluated (e.g. biomaterial volume, porosity, pore size).

Figure 5-1: Major components of standard µ-CT scanner.
The x-ray generated by the micro-focus tube passes through a filter to reduce the amount of x-ray that will be attenuated. Once the x-ray passes through a rotated object, it will be recorded by a 2D charge-coupled detector.



5.2.4 Three-points bending mechanical tests

Mechanical tests can measure the strength (maximum force), stiffness, rigidity, and toughness of the healing bones as healing outcomes, and although maximum force to fracture and toughness can only be measured once, we can obtain multiple measurements for stiffness and rigidity. The mechanical properties of healing bones are usually estimated by using the load in torsion or the three-points bending mechanical tests, depending on technical and physiological considerations. In vivo, long bones are exposed to bending and torsion forces all the time; therefore, it is logical to test in these mechanical properties in healing long bones. Due to the variability in the fracture alignment and the asymmetry of the fracture callus, tension and compression tests are not recommended (187).

The three-point bending test provides the distribution of internal loading to study the mechanical characterization of the fracture site in a physiological manner (188). The bone sample is fixed to

the testing machine, and the load is applied until the bone is broken (Figure 5-2). At the start of the three-points bending mechanical test, the bone is not in contact to the loading point (zero displacements), then the loading point contacts the bone with a small preload force (< 1 N). The values of the loading force and the displacement is recorded and stored in a predefined file while the loading point is moves downward with increasing force. Interpretation of these data combined with the morphological geometric data of the object from the μ CT analysis provides an estimation of the structural (mechanical) properties of the bone sample.

The mechanical properties illustrated in Figure 5-3 are structural, rather than material properties. The mechanical properties of the bone or the healing callus depend on the quantity and the density of the mineralized tissue, which can be measured and estimated through histological and radiographical examination (188).

Figure 5-2: Three-points mechanical bending

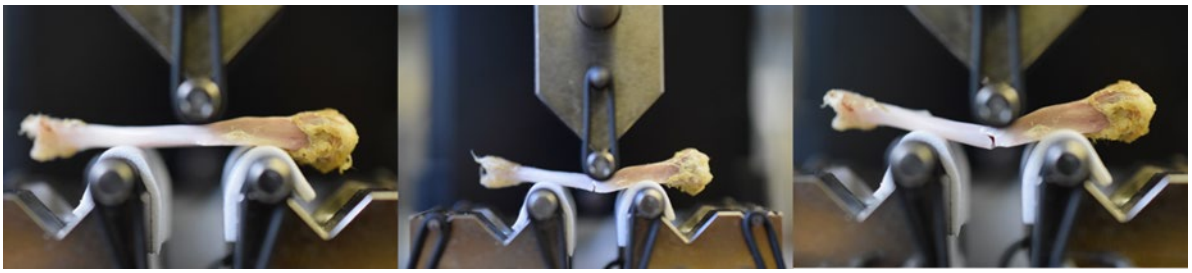
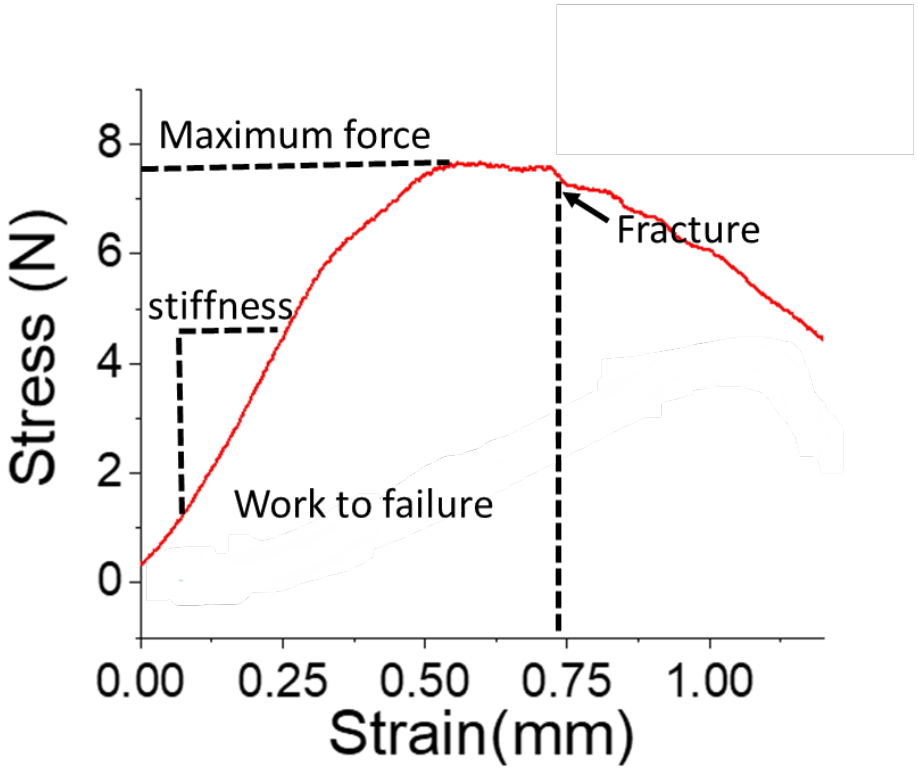


Figure 5-3: Example of a mouse tibia load-displacement curve



5.2.5 Histological assessment

Histological and histomorphometric assessment of the healing bone is essential to understand and describe the mechanical and structural quality of the healing callus. The healing callus is a repair tissue that follows and remodels after a fracture. The healing callus contains cartilage, bone, fibrous and hematopoietic tissues, thus histological assessments should be able to asses the heterogeneity of this tissue (187).

The histomorphometric analysis is an essential outcome assessment of the healing bone to explain the mechanical and morphological findings by µ CT and mechanical bending tests.

Staining of the healing tissue is one of the applied methods to facilitate the visualization and the differentiation between different cell types and tissue types mainly between cartilage and bone within the healing callus.

Proper histomorphometric analysis required the correct selection of anatomical plane of the tissue sectioning, in addition to the sampling, embedding, fixation and tissue staining (34).

The histological processing of bone samples can be performed in two ways either leaving the bone minerals and this is call uncalcified process or after decalcification. The decalcification process of bone samples is conducted by either using EDTA acid or formic acid in a Paraffin sectioning/ embedding (189). The paraffin embedding, or sectioning is suitable for most of the histological stains after embedding the sample in paraffin and cutting the sample into 4 µm thickness through cortical or cancellous bone. Paraffin embedding/ sectioning is the suitable method for immuniohistochemistry analysis because the materials used in this sectioning less destructive to bone's proteins than the ones used in the uncalcified sectioning (189). Two limitations associated with the use of paraffin embedding technique; firstly, is the time need to complete the decalcification process which may take weeks or even months depending the sample size (189). To accelerate the process, we can use higher concentration of the acid, but this can lead to some distortion of the bone sample. The second limitation is related to the quantitative measurements for the histomorphometric analysis, as the paraffin sectioning can lead to up to 15% shrinkage or distortion in comparison to only 10-12% distortion for the plastic embedded samples (190). The second sectioning method is to use plastic material to embed bone sample without decalcification. One of the most use plastic material is methyl methacrylate (190).

The uncalcified process allows faster time to process larger samples. Also, this processing method provides thin sections that permit the evaluation of the bone structure and cellular analysis with its fluorochrome labelling. Despite high technical skills and advanced equipment needed for uncalcified processing, but this process allows for excellent visualization of the bone cells and proteins in an intact specimen (190).

Types of stains that have been used to assess fracture healing are:

- Alkaline phosphate stain highlights osteoblast cells and not osteoclasts. It has been shown that the quality of alkaline phosphate stain decreases with formalin fixation.

  Osteoblast cells during proliferation show alkaline phosphate activity which in return will be a good marker that can be easily detected by using certain materials such as nitro blue tetrazolium which subsequently stains the cells as blue-violet when the alkaline phosphate is present (191). After sectioning, the samples are gradually hydrated with alcohol series, then incubated in alkaline phosphate substrate solution for about two hours. After washing the sections with distilled water, the samples later are treated with counterstain (191).
- Von Kossa staining demonstrates the calcium salts (e.g., carbonate and oxalate) and phosphate components of the bone mineral. In this method, the bone sections are deparaffinized and then hydrated with alcohol for five minutes, then the sections are washed with distilled water. Subsequently the sections are treated and incubated with silver nitrate solution, and the sliver will deposit and replace the calcium salts, which will be then recognized by their metallic sliver stain. Calcium deposits will be stained black or dark brown (192).

- Staining for activity of Tartrate-resistant acid phosphatase (TRAP) is used to define osteoclasts due to their high expression of this enzyme. TRAP is metalloprotinase that is associated with hematopoietic lineage cells activity, especially osteoclasts and human macrophages (193). Osteoclasts cell secret TRAP during its bone resorption behavioural activity, so it is a good marker for active osteoclast cells (194).

  The paraffin sections of the samples are deparaffinized by xylene and 70% ethanol then hydrated to water. The sections placed in prewarmed TRAP solution which are then incubated and rinsed with distilled water, and later they are counterstained by Fast Green. Osteoclast cells will be stained and look bright red on a green background (193).

To compare the stain intensity of distinct cell profiles in the experimental or treatment groups, we use a special morphometric image analysis software acalled ImageJ(R), which is available in the public domain. The images from the histological slides need to be analyzed. ImageJ can be used to analyze the whole image or region of interest from the slide. For example, the investigator who wants to count a number of cell profiles will click to select the positive and negative nuclei of a selected area (region of interest) as a representative area of the sample or the whole image, the program will automatically generate immunohistochemistry index (195). The results will be tabulated in a table and can be exported to an excel sheet later.

This method is associated with selection bias that needs to be reduced by following a reference sampling plan to be followed, which includes all the regions of the slides, the investigator reviewing the microscopic slides would be masked as to the group-origin of the sample (195).

5.2.6 Molecular Measurements

5.2.6.1 Multiplex Luminex cytokine analysis

The immune system uses specific molecules called cytokines to send signals between each other. These cytokines are of particular importance for the recruitment of immune cells to the site of an invading pathogen, especially after injury (196, 197). The identification of different cytokines is vital to understand the pathological and the physiological immunological processes that occur in a living organism and can be used as a biomarkers for many diseases (197). They are classified as proteins, glycoproteins, and peptides depending upon their composition.

The new technology such as Luminex Multi Analytic Profiling (xMAP) allows us to simultaneously identify and quantify different secreted proteins such as cytokines, chemokines or growth factors. This technology introduced invaluable advantages to the biomedical research because of its efficiency, speed and cost effectiveness (198).

In general, Immunological interactions involve a wide range of cytokines which can be regulated up and down based on the function of each cytokine. It is therefore essential to have full picture of the cytokines that are involved in the immunological response or reaction such as inflammation to understand the undergoing process.

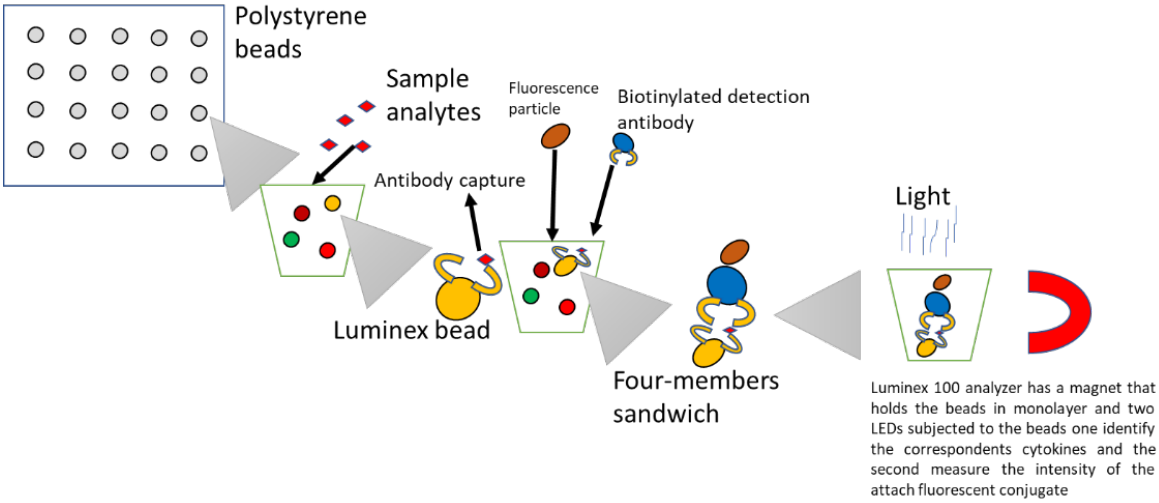
Tradition cytokine analysis technologies such as the enzyme-linked immunosorbont essay (ELISA) are not practical because they can only test for one cytokine at a time, and this is why multiplex systems where developed for cytokine analysis.

The essential component of this Luminex xMAP technology is the polystyrene or the microspheres beads which are combined in a single well of 96-well microplate-format assay, and each bead in the set reacts to the specific antibody of one of the interested cytokines from the

targeted sample (such as blood serum). The beads are dyed or precoated with specific antibody analyte capture, for the specific molecule of interest (199). These dyes provide each bead with a unique spectral code, and when it is added with a sample (such as blood serum), the specific molecules on the surface of these beads will react to its designated analyte from the sample. A biotinylated detection molecule (antibody specific) is then added, and this will form an antigen-antibody complex. Then, a conjugated fluorescent material is added which will bind with the biotylinated detection molecules associated with the certain cytokine on the beads forming four-parts solid complex. The sample is run through an instrument such as Luminex 100^{TM} that analyzes the spectral address of the microsphere beads and the amount of the associated florescence; subsequently the concentrations of many cytokines within one sample can be determined (59)(Figure 5-4).

In conclusion, Luminex technology is suitable for identifying changes in the expression of different pro- and anti-inflammatory cytokines and growth factors from one sample using one of the Luminex assay (198).

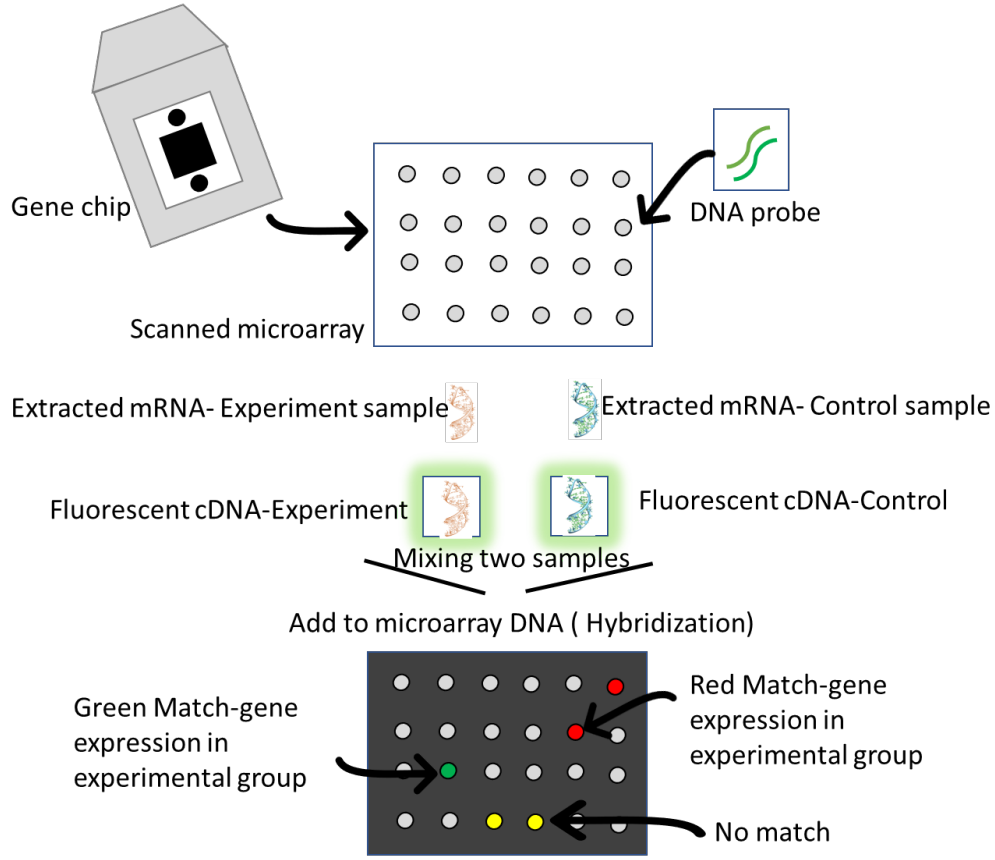
Figure 5-4: multiplex Luminex cytokines analysis process



5.2.6.2 Gene expression microarray

Gene expression microarray is a procedure that provides information about the transcriptional activity of different genes in biological samples (187), allows us to identify and assess the expression of thousands of genes in single process. Gene chips or DNA microarray slides are of microscopic solid surfaces printed in specific locations with thousands of minute spots; every spot has a recognized DNA sequence of certain gene called a probe; this probe can detect An expressed set of messenger RNA (mRNA) transcripts which are expressed through a gene or a group of genes (Figure 5-5) (200).

Figure 5-5: Microarray gene sequencing



Microarray experiemnts involve: a) collecting and extracting RNA from the experimental, and the the reference samples; b) the RNA molecules are converted into complementary DNA or (cDNA); c) cDNA of each sample is then dyed by different fluorescent dye, for example, red for the experimental group and green for the reference; d) then the two cDNA samples are mixed and hybridized within microarray slides. Hybridization is the process of binding the mixed cDNA sample to the DNA probes of the microarray slide. e) After that, the slide is scanned to detect and measure the expression of each gene. So if the gene is expressed in a higher level in the experimental group the associated spot on the slide would look red, and if the gene expression

in the experimental group is lower than the control group the spot colour would be green, and if there is equal expression in both groups then the spot would appear yellow (Figure 5-5) (201).

The advanced knowledge obtained from sequencing the entire genome of many species has significantly contributed to studying the transcriptome of different processes that can be assessed by microarray analysis (187). It is advisable that these procedures to be done in established institutes or centers with a tracking record of conducting this kind of studies, these centres usually have the equipment and the instruments, expert technical staff and the necessary training resources.

General considerations and steps needed for conducting microarray experiments:

i) It is suggested to have at least three replicates for each experimental group, which will allow for sufficient amounts of RNA to perform the microarray and overcome any technical problems.

ii) The lab should be able to identify any artifact from the microarray hybridization or issues related to the chip manufacturer through a clear quality control procedures.

iii) Posthoc bioinformatics analysis, is crucial to identify differentially expressed genes, followed by interpretation of the differentially expressed genes according the available biological knowledge.

iv) Since microarray studies are expensive, it's recommended to collaborate with computation or bio informatic statisticians with an extensive record of gene expression analysis.

5.2.7 Surgical models and procedures

Bone regeneration and healing after fracture surgery is a complex process, and non-union or delayed healing of the patient's fractured bone remains a primary concern (18). In vivo animal models can help to investigate the complex fracture healing process, which may not be possible to investigate in vitro(202). The choice of animal model depends on many factors, the most important of which are the research question and the biological process to test, in addition to the anatomical variability. During earlier periods, large animal models such as dogs, sheep and rabbits were used to investigate fracture bone healing (202). The use of these models is justified by the volume of the bony structure in these animals and the similarity to the human bone, specially the presence of the Haversian remodelling system (203). However, these models are challenging to handle, the cost for the husbandry is excessive, and it is extreme difficulty to obtain transgenic models for these animals (202). Since the 1990s, the scientific community has promoted rat fracture healing models, due to low cost, easiness of animal handling, short breeding cycle and faster regeneration. However, the genetic modification of rats is somewhat limited.

Nevertheless, the small animal models have similar remodelling system like that in larger animals (203). Moreover, the development of techniques to stabilize fractured bones in small animals has provided a significant boost to consider rodent models as suitable to test fracture healing (202). Although the bone structures of the human and the mice are different, the healing phases of the bone fracture are very similar, which makes the mouse model a good one to use in fracture healing research (204).

Additional factors to consider in selecting the model are the sex and age of the animal and the type of surgical fracture model. The age of animals affects the healing outcome (202) and that the time required to heal increases with age. Adolescent animals (rodents) need four weeks to bridge the gap, while older animals require 26 weeks for bone tissue healing (205). Data from a study on mice fracture healing has shown that osteoblast cells in old animals showed a delayed and decreased response to osteogenic stimuli which results in impaired endochondral ossification (206). The selection of gender will also depend on the research question; recent studies support the inclusion of both sexes in experimental trials whenever possible, especially when measuring pain as an outcome (207). Female animals may be a good model for osteoporosis, and for other bone diseases, nevertheless, their physiological and biological reaction toward drug administration should be taken into consideration when using them in fracture surgery (202). Studies that have shown that the sex-relating variations in drug pharmacokinetics properties in mice mimic the ones observed in females humans (11).

Another important consideration for fracture healing research is the type of fracture, which could be categorized into:

I) Simple fracture models are used to estimate the effect of different interventional medications and genetic modifications in fracture healing (208). This model can be further classified based on the anatomical variations, such as fibular, femur, or tibia fracture (long bones). The simple fracture can be performed either as a closed one, which is usually created using three-points bending equipment; or as open fracture surgery. The second approach allows the fracture to have a standardized position and location and is usually stabilized with intramedullary pins (204). These models are

usually done in the tibia because unlike femur, is associated with very thin layer of soft tissue (202).

II) Critical size bone defect models have been used in research to address the clinical scenarios, that may lead to a delay in bone union or non-union. Therefore, these models are often used to test and evaluate the effect of growth factors that promote bone repair as well as bone regeneration biomaterials (208).

Using mice with altered genes, this model also provides researchers with an excellent platform to study the effect of different signalling pathways in bone regeneration (208). This model can be further classified into drill-hole or critical size defect models (204).

III) The ectopic bone formation model has been defined as the model in which bone formation occurs in sites where the bone is not supposed to grow (208). These models are subcutaneously or intramuscularly and are typically used to assess osteoinduction of grafts with stem cells therapy or growth factors (209).

5.2.8 The methodology used in the second manuscript

5.2.8.1 The effect of surgery time on bone fracture pain and healing

To assess the effect of surgery time on postoperative pain and bone healing, we used a tibia fracture model in mice, in which animals underwent bone fracture surgery at different times of the day. We investigated the behavioural indices of pain, including responses to the weight-bearing and guarding tests, as well as the biomechanical and histomorphometric properties of the healing bones.

- Animal model

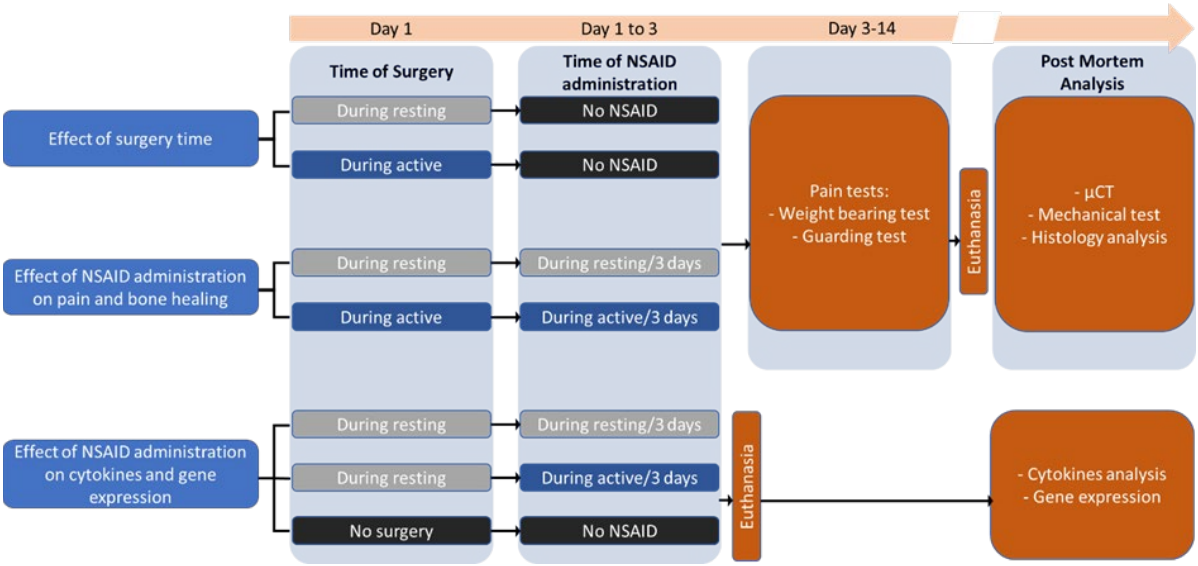
After receiving the approval of the Animal Care Committee at McGill University, 4-months-old wild-type C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were used in this experiment. We used this strain because it is deficient in melatonin, which helps to identify the pathways independent of the melatonin systemic effect (210). The mice weighed 25 to 28 g and were housed in pathogen-free conditions at 22°C with a 12-hour alternating light/dark cycle fed on water ad libitum.

- Experimental groups

Cage assignment, mouse numbering and allocation to study group were performed by a researcher not otherwise involved in the study procedures. Random group assignment was done following an automated process using the Experimental Design Assistant from the National Centre for the Replacement and Reduction of Animals in Research. Before the surgical intervention, mice were acclimated for two weeks to their new environment and exposed to behaviour testing equipment twice before obtaining baseline measurements.

Mice were randomly assigned one of two study groups. One group received open tibia-fracture surgery at zeitgeber time ZT3, where ZT0 refers to the time the lights turn on in the animal facility, which is the beginning of resting time in mice. The second group had the same surgery at ZT13, the beginning of the active phase, during which the light is off. Mice are nocturnal animals for which the activity phase starts at the beginning of the night and ends in the morning (Figure 5-6).

Figure 5-6: Diagram of the objectives of each experimental trial

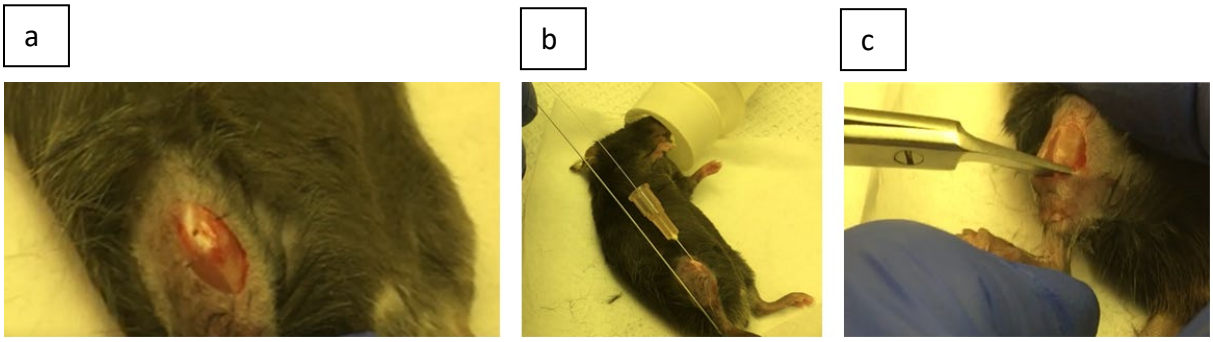


- Surgical intervention

For our study, we used the open osteotomy fracture model developed by Grestenfield et al. (32). All surgeries were performed by the same operator who was not involved in the randomization, allocation concealment process or outcome measurements. Anesthesia was induced initially with 4% isoflurane/oxygen mixture and maintained at 2%, and a buprenorphine (5 %) subcutaneous injection for pain control was given before surgery. An incision centred over the knee joint medial to the patellar ligament was done to create an entry portal to the tibia medullary canal using a 27-gauge x ½ inch TB syringe. The stylet of a 25-gauge spinal needle was inserted in an intergrade fashion through the lumen of the 27-gauge needle down to the distal growth plate, and its tip was used to bend the spinal needle wire, which was cut 1 mm above the bent then it was inserted and adapted smoothly beneath the patellar pliers. The wound was closed with 5-0 absorbable

vicryl suture. Mice were monitored for any signs of improper healing or infection. All animals were euthanized two weeks later at the same time of the day (Figure 5-7).

Figure 5-7: Pictures of the open tibia fracture surgery.
a) skin incision over the patellar ligament (white tissue), b) insertion of both the gauge needle followed by the spinal needle for internal fixation, c) snap and sharp fracture of the tibia bone.



- Pain assessment

Behavioural indices of pain, including weight bearing differences and guarding, were assessed at baseline (pre-surgery), and on day 3, 7 and 14 after bone fracture surgery. We performed the assessments at the same time of the day between ZT5 – ZT7 and the investigator responsible for performing the assessments and collecting the data was blinded to experimental design and different intervention groups.

Guarding behaviour test (GBT)

Each mouse was placed individually on an elevated stainless-steel mesh. Both paws (of the fractured and non-fractured legs) were carefully observed for one-minute periods every five minutes over one hour (12 measurements). A score of 0, 1, or 2 was given according to the postural position of each paw in the one-minute scoring periods. Score 0 was given if the paw

skin was blanched or distorted by the mesh (indicating weight bearing); and score 2 meant that the paw was entirely off the floor; score 1 was given if the paw touched the floor without blanching or distorting. After the 1-hour session, the sum of the 12 measurements (which could range from score 0 to 24) given during the session was calculated for each paw. Subsequently, the scores of the fractured and non-fractured legs were compared between different experimental groups (181, 211) (Figure 5-8).

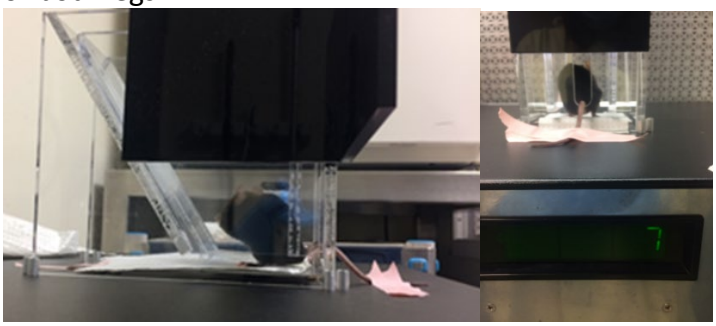
Figure 5-8: Picture of the guarding test mesh.

Weight-bearing test

The relative distribution of the weight that the animal bears on the injured and uninjured legs was determined by an in-capacitance meter (IITC.inc, CA, US). The device contains a small Plexiglas chamber (11.0 × 19.7 × 11.0 cm) with floor weight plate sensors. The device records the weight that each limb exerted in grams. Each mouse was positioned in the angled chamber so that each hind paw rested on a separate weighting plate. The weight exerted by each hind leg was measured for 5 seconds and then averaged. The change in paw weight distribution before and after the surgery was calculated by estimating the difference in weight (g) between the left

(control) and right (fractured) legs (212). This difference was used as a pain index in the fractured leg (Figure 5-9).

Figure 5-9: In-capacitance meter chamber with the mouse inside to measure the weight-bearing of both legs.



- Microcomputed tomography (µ-CT) assessment

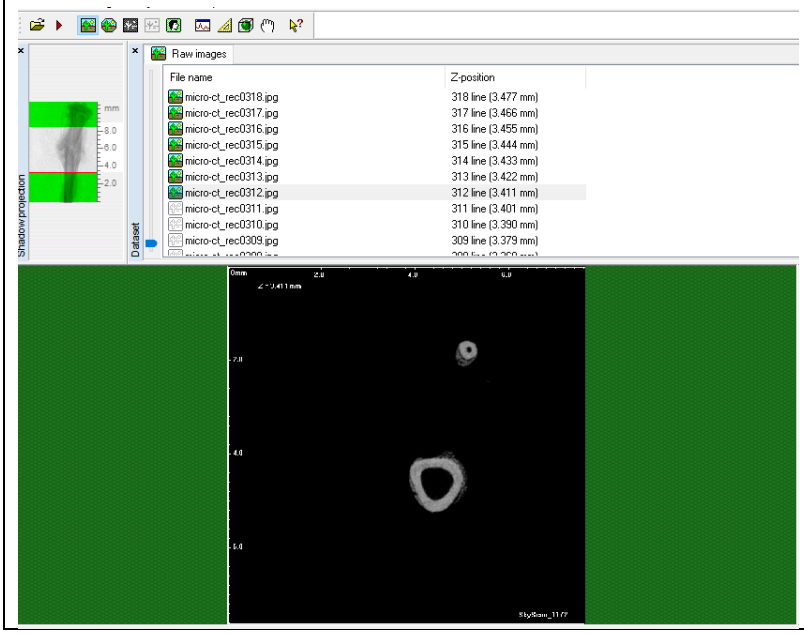
The bones harvested at day 14 were scanned using Skyscan 1172 micro-CT scanner (Skyscan 1172; Bruker-microCT, Kontich, Belgium), the samples were inside small plastic tubes with 70% ethanol (Figure 5-10). Double blinding was implemented during the scanning process and the 3D reconstruction. Scans were taken with a 5.88 µm pixel size, at scanner voltage and current set to 59 kV and 167 lA, respectively. Image stacks can be processed via CTAn software and 3D segmentation software CTVol and CTVox (Bruker, Kontich, Belgium) and via BoneJ, an online tool that can be downloaded for free.

3D reconstructions were created using CT-Analyser software (Bruker micro-CT, Kontich, Belgium) to measure the callus size, total volume (TV) and bone volume (BV). The bone volume fraction (BV/TV), and the trabecular number and thickness which were calculated for a volume of interest, that encompass the fracture callus within a 1.5 mm (255 slices) vertical range, centred on the

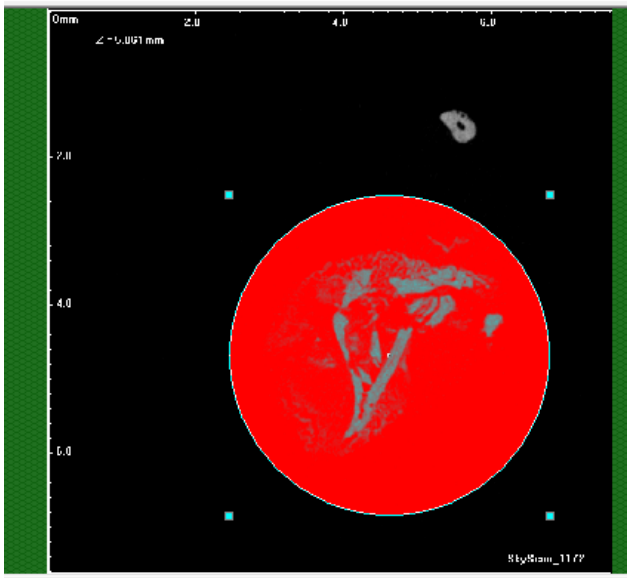
osteotomy. The region of interest (ROI) for each section was selected as the outer boundary of the fracture callus, excluding the fibula. A binary threshold gray level of 70/255, corresponding to the mouse trabecular bone, was used to segment mineralized bone from soft tissue (65, 81, 83, 213) (Table 5-2).

Table 5-2: µ-CT 3D reconstruction process

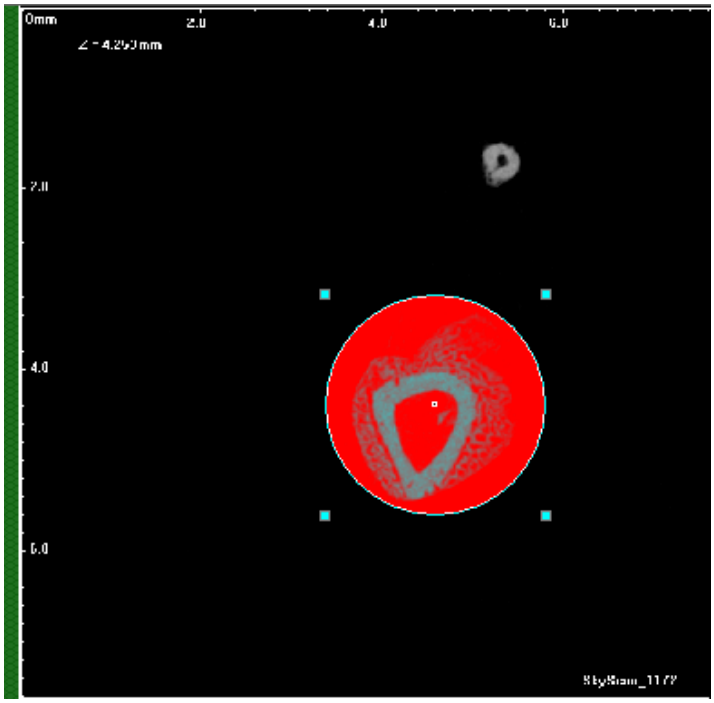
1- Open CTAn software (CT-Analyser; Bruker micro-CT, Kontich, Belgium).
2- Click the folder button; load reconstructed CT data.
3- On raw image, select the top and bottom sections (from the fracture center, approximately ±2mm, end of bone callus).



4- Select the biggest callus area, click on Region of Interest (ROI), and choose all callus area

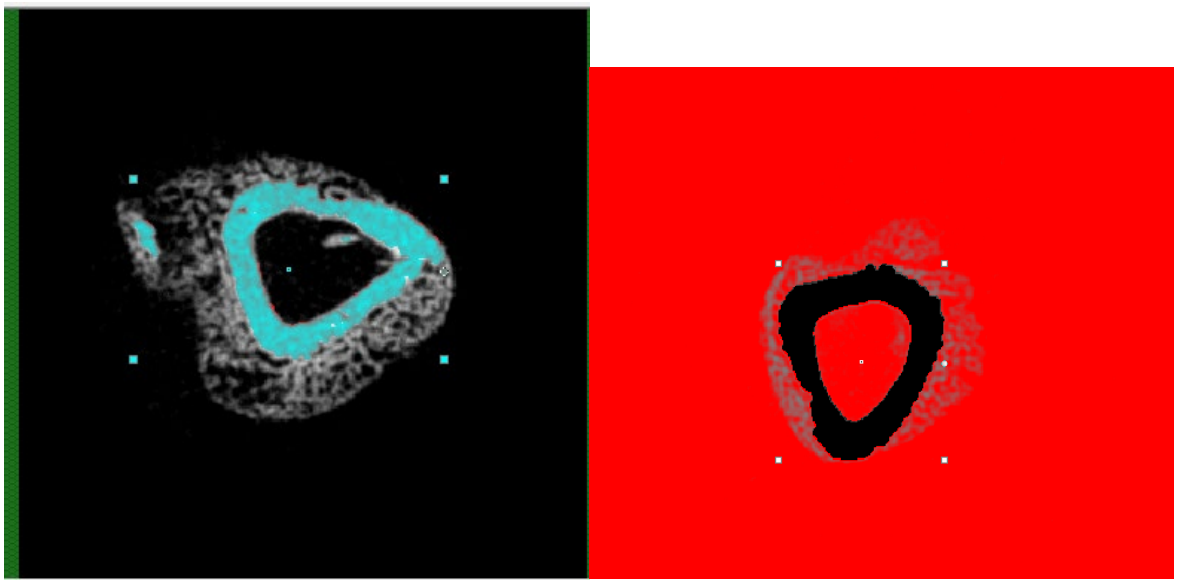


5- Check whether all the callus is included within the ROI. Refine the ROI when moving up or down to all the section.

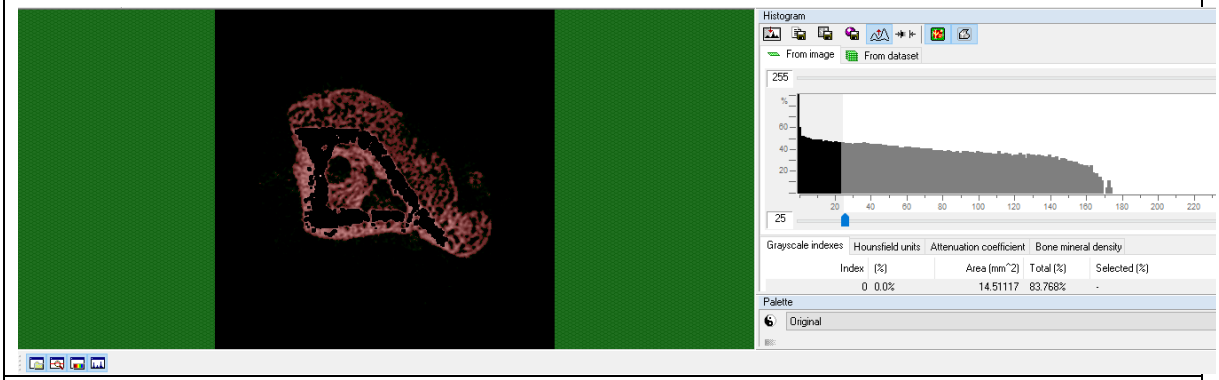


6- Binary selection to remove all the cortical bone in the callus tissue

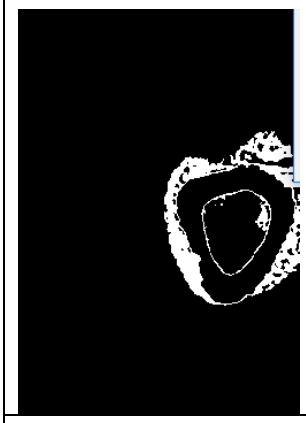
7- Custom processing, then we see the cortical bone ROI image overlapped with our current ROI



8- Binary selection, and this time all the cortical bone is removed as in the picture below.

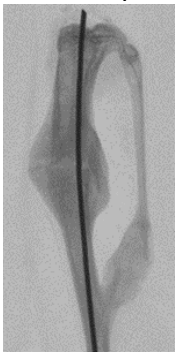


9- Save and reopen the binary data.



10- Custom processing; open the internal tab and choose 3D analysis for BV/TV, Trabecular bone number, thickness and spacing.

Figure 5-10: µ-CT image of the fractured tibia after 14 days before removing the pin for histomorphometric analysis.



- Biomechanical testing

The biomechanical properties of the tibia healing callus were determined by loading the right tibia to failure in a three-point bending test which was conducted using a Mach-1 mechanical tester machine (Biomomentum, Laval, Quebec), as previously shown in Figure 5-2. This type of test was chosen based on a previous study (214). The distance between the supports with the bending fixture was 10 mm, and the diameter of the supports and loading nose was set at 0.25 mm. The machine applied downward bending load to the fractured tibia (over the fractured site) at a rate of 0.016 mm/second until failure. A load-displacement curve generated using Mach-1 software (Tempe, Arizona) was used to determine three parameters: stiffness (N/mm), ultimate force (N), and work to failure (N*mm).

- Histological analysis

We dehydrated the bone defect samples (right tibiae) in ascending concentrations of ethanol (70–95%) and embedded them in methyl methacrylate at the Bone Biology Lab, McGill University. After polymerization, three subsequent 6-µm-thick sections, crossing through the middle of the defect, were obtained from each sample and stained with either tartrate-resistant acid phosphatase (TRAP), Masson's-trichrome or Von-Kossa stain to assess osteoclasts, collagen, and mineralization, respectively. For each sample, we analyzed one histological section per stain. Images of the histological slides were produced using an optical microscope (Zeiss-Microscopy, Jena, Germany). We defined the region of interest for osteoclast, collagen and mineralized tissue histomorphometry as the area of the histological section enclosed by the cortical margins and the borders of the healing callus of the fracture site. Osteoclasts were calculated using ZEN-2012-SP2 imaging software (Zeiss-Microscopy, Jena, Germany), and the data was demonstrated as

osteoclasts number per square millimetre of mineralized tissue (OC/mm2). We analyzed the percentages of mineralized tissue and collagen in the region of interest of the healing site using ImageJ v1.45 (Wayne Rasband; NIH, Bethesda, MD, USA) and documented as mineralized tissue percent (MT%) (78, 215). Histomorphometric analysis was performed by the (Lina Abunada), who was blinded to group allocation.

5.2.8.2 Effect of the timing of NSAID administration on pain and bone healing

To assess the effect of the timing of NSAID administration on pain and bone healing, we performed a surgical intervention as described above. We used sixteen mice in this experiment, which were housed and handled as described above.

- Experimental groups

Two groups of mice assigned randomly; one group received fracture surgery and postoperative subcutaneous injections of carprofen 20mg/kg only at resting time ZT3 for three days, whereas the second group had the same treatment at the active time ZT13. The carprofen dose used is equivalent to a typical postoperative prescription for humans of 500 mg every 8 hours of ibuprofen (1500 mg/day) (216). Mice were observed for any signs of improper healing or infection. All animals were euthanized two weeks later (Figure 5-6).

- Pain assessment

The methods of assessment were carried out as in the previous experiment.

- Assessment of the healing bones

The preparation of the bone samples and the measurements were performed as in the previous experiment.

5.2.8.3 Identification of inflammatory cytokines and metabolic pathways affected by the timing of NSAID administration after three days of bone fracture surgery

To address this aim, we conducted Luminex cytokine analysis and RNA microarray sequencing of the callus three days after surgery to identify the affected inflammatory and metabolic pathways.

- Animal model

We used nine mice to address this aim, housed and handled as in the previous experiments.

- Surgical intervention

The surgical intervention was performed as in the timing of NSAID administration experiment for all the groups at the same time of the day.

- Experimental groups

Mice were randomly assigned to three groups; one received NSAID after the surgery during the resting phase of the mice ZT3, the second group received NSAID at the beginning of the active phase at ZT13, and a third group did not receive any surgery or NSAIDs. Mice were observed for any signs of improper healing or infection after surgery. All animals were euthanized after three days (Figure 5-6).

- Assessment of early healing at the molecular level

Cytokine profile in blood serum

Blood serum was collected from each mouse at day three after the surgery, and frozen at -80°C in sperate vials until further analysis. For cytokine analysis, the serum samples were thawed at room temperature. A multiplex inflammatory cytokine kit (Cytokine Mouse Magnetic 20-Plex Panel, Thermo Fisher Scientific, Waltham, MA) and Luminex 100/200 multiplexing instrument

(Luminex Corp., Austin, TX) were used to analyze the inflammatory cytokine profile of each serum sample. The Cytokine Mouse Magnetic 20-Plex kit was used for bead assays of FGF basic, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p40/p70), IL-13, IL-17, IP-10, KC, MCP-1, MIG, MIP-1α, TNF-α, and VEGF. These assays were performed following the manufacturer's protocol. The Luminex 100/200 System was used in combination with xPONENT instrumentation software for measurements and analysis. The experiments were conducted in triplicate, and the mean fluorescence intensity (MFI) for each cytokine was used to calculate its concentration (59, 217).

RNA isolation and microarray

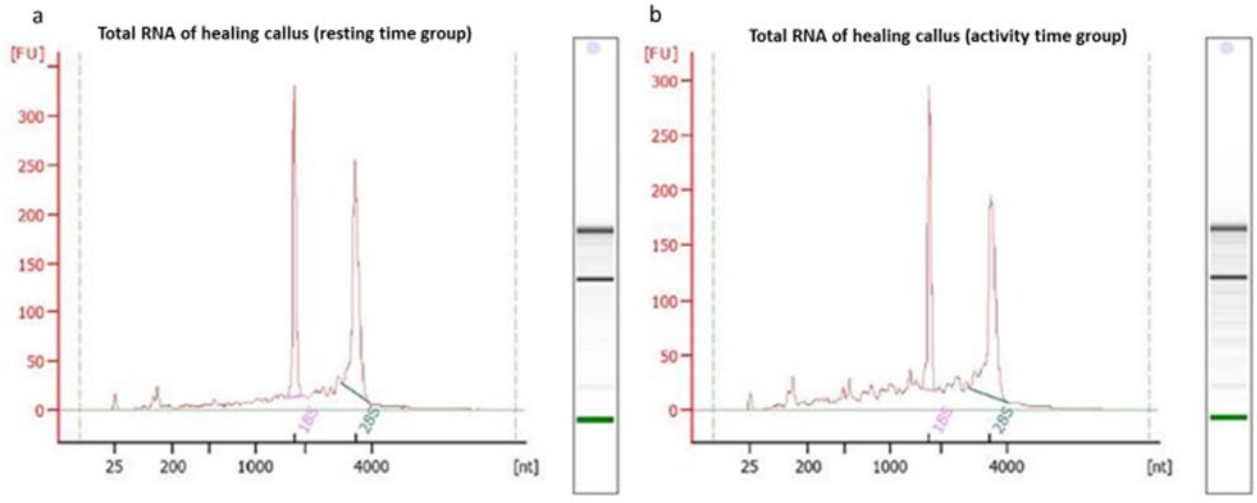
The central one-third of the fractured tibia including all the healing callus and hematoma was harvested on day three post-fracture from the experimental groups and stored in Alloprotect (Qiagen, Toronto, Ontario). For reliable gene expression analysis, including microarray analysis, the immediate stabilization of RNA and proteins in biological samples such as the healing callus of the bone is an absolute prerequisite. Without stabilization, directly after harvesting the samples, changes in the gene expression pattern occur due to specific and nonspecific RNA degradation and transcriptional induction. Alloprotect Tissue Reagent prevented these changes by providing immediate preservation of DNA, RNA, and proteins in animal tissues for up to 1 day at 37°C, 7 days at 15–25°C, or 6 months at 2–8°C, allowing storage, transportation, and processing of samples without ice or dry ice (alternatively, the samples can be archived at –20°C or –80°C) (218).

Subsequently, bone pieces with the hematoma were homogenized using the Precellys 24 homogenizer (Bertin instrument, Montigny-le-Bretonneux, France. The lysing tube used for

homogenization was CKMix50_2mL with ceramic beads and 1 ml of QIAzol (Qiagen, Toronto, Ontario) buffer. We used the following protocol: i) speed at 6500 rpm, ii) 2 cycles of 20 seconds with a 30-second break between cycles. The process was done at a temperature between 0 and 4°C, controlled using the Cryolis cooling system.

The total RNA was isolated from bone tissue using QIAzol (Qiagen, Toronto, Ontario) and then purified using RNeasy Lipid Tissue Mini Kit columns (Qiagen, Toronto, Ontario) following the manufacturer's instructions. The extracted total RNA was sent for Whole-Transcript Expression Analysis at the McGill Quebec Genome Center. Total RNA was quantified using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc., Waltham, MA) and its integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies, Waltham, MA) (Figure 5-11).

Figure 5-11: Representative Bioanalyzer electropherogram profile of the RNAs contained in the healing callus after open tibia fracture surgery and three days of NSAID administration either during the resting time (a) or activity time (b).
The electropherogram shows the size distribution in nucleotides (nt) and fluorescent intensity (FU) of total RNA. The most dominant peaks are 18S and 28S. Associated gel images are shown alongside the plots.



Sense-strand cDNA synthesized from 100 ng of total RNA, and fragmentation and labelling were performed to produce ssDNA with the Affymetrix GeneChip WT Terminal Labeling Kit (Thermo Fisher, Waltham, MA) according to the manufacturer's instructions. After fragmentation and labelling, 3.5 µg DNA target sample was hybridized on the GeneChip Mouse Gene 2.0 ST array (Affymetrix, Waltham, MA) and incubated at 450°C in the GeneChip Hybridization Oven 640 (Affymetrix) for 17 hours at 60 rpm. GeneChips were then washed in a GeneChip Fluidics Station 450 (Affymetrix) using the Hybridization, Wash, and Stain Kit (Affymetrix) according to the manufacturer's instructions. Finally, the microarrays were scanned on a GeneChip Scanner 3000 (Affymetrix). The data were normalized, and the analysis was only included the identified units within the samples. The analysis was conducted using GeneSpring GX 10 software (Agilent, Santa-Clara, CA, United States). To identify genes differentially expressed between groups of mice that received NSAID during active versus resting time and control groups, we used unpaired Student's t-tests to compare between groups. The genes that presented an up- or down-regulation with p-value < 0.05 were identified. To avoid loss of substantial numbers of true positive genes, no correction was performed for multiple testing.

Using the Gene Ontology (GO) database (PANTHER Classification version 11), the PANTHER Overrepresentation Test was performed on the list of genes that were differentially and significantly expressed (as described above) to identify the functional biological processes involved in the genes. Specifically, this binomial test determines whether there is a statistical overrepresentation or underrepresentation of the genes that expressed differently between the active versus resting time group relative to the reference list in the GO ontology database for the mice species (a full list is published in the Gene Expression Omnibus (GEO) data repository,

available upon request). Using the PANTHER Classification System, a 'gene ontology' classification of the genes/expressed sequence tags (ESTs) that are significantly ($p < 0.05$) up- or downregulated was performed to compare between different experimental groups. We classified differentially expressed genes into various biological processes to assess their functional significance, then calculated the proportional distribution of genes in each process.

The list of genes that significantly differentiated between active versus resting time groups were employed to identify significantly activated pathways by comparing their functional annotations according to the PANTHER Classification System (www.pantherdb.org) with the whole mouse genome (data updated to NCBI's January 10, 2011 release) by the binomial distribution function (219).

5.3 Randomized controlled trial

A randomized controlled trial (RCT) is the proper design to conduct clinical studies. RCT used to investigate the efficacy of a new drug or medical intervention in a patient population (220). One key strength of the RCT is the random allocation of the participants to the treatment(s). In addition, the RCT contains control groups either receiving no treatment (a placebo-controlled study) or a previously approved treatment (a positive-control study) or gold standard care. It is well accepted and recommended that the main RCT should be preceded by a pilot study to provide feasibility information about important aspects of the study, including the recruitment rate and whether it is possible to measure the suggested outcome with a good ratio of compliance from participants during the determined follow-up period. The pilot trial will mimic the main one and ensure that the main trial will deliver the maximum benefit (221).

The strengths of the RCT design can be summarized as follows: 1) It provides the most reliable evidence for the efficacy and side effects of medical interventions; 2) Incidence or prevalence can be assessed since the measurement is conducted in patients who were randomly selected from the population; and 3) It has the least bias due to the randomization and controlling of the confounders.

However, the RCT design also has weaknesses: 1) It is relatively expensive and time-consuming to perform; 2) It could pose ethical issues, for example when some individuals are required to accept the risk to assess certain conditions that may not benefit them; 3) It is inefficient in assessing delayed outcomes; 4) Its findings might not apply to the population (risk of generalizability) since the selected patients, despite the random process, may not be good representatives of the total population.

5.3.1 Methodology used in the randomized clinical trial

5.3.1.1 Study location

The current project is a single centre, double-blind, RCT. Randomization took place at the individual level. We carried out the study in the Dental Teaching Clinics at the Jordan University of Science and Technology in Irbid, Jordan.

5.3.1.2 Ethical approval

The study obtained approval from the Institutional Review Board of Jordan University of Science and Technology (protocol number 393/2017) (Appendix III).

5.3.1.3 Registration

The RCT is registered in the clinicaltrial.gov clinical trial registry (No. NCT03789058).

5.3.1.4 Study participants

For recruitment, which began in July 2018, we approached subjects who were referred for third molar extraction at the Oral and Maxillofacial Surgery (OMFS) Department, Jordan University of Science and Technology, Irbid, Jordan. The maxillofacial specialist examined all patients clinically and evaluated their radiographs to determine their suitability for inclusion in the study. Patients who had been indicated for third molar extraction and fulfilled the study criteria (described below) were invited to participate in the study. Those who agreed signed a surgical procedure consent form (Appendix III) on the day of the surgery.

5.3.1.5 Inclusion and exclusion

The study includes adults scheduled at the OMFS dental clinics for surgical extraction of third molars, restricted to lower third molars that are bony impacted to standardize the clinical cases. To minimize the effect of differences in bone impaction depth and tooth angulation on the outcomes, the surgeon used a validated classification for impacted teeth to evaluate the radiographs (222).

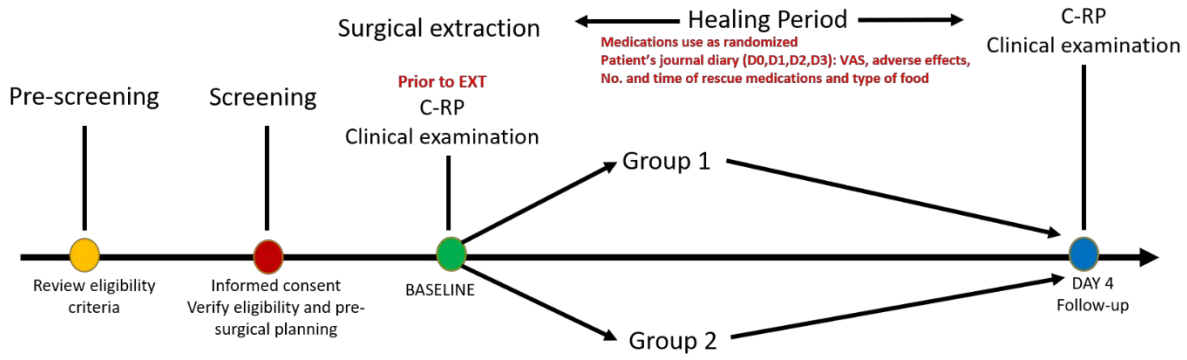
Patients were eligible for the study if they (i) were aged between 18 and 35 years, (ii) were healthy according to the American Society of Anesthesiologists classification (subject have no active infection, trismus, hyperthermia, or swelling before surgery and must be able to maintain adequate oral hygiene), (iii) had an adequate understanding of written and spoken English or Arabic, (iv) were capable of understanding and completing the inform consent and study questionnaires, and (v) agreed to fully adhere to study instructions. The upper age limit was set to minimize the risk of undiagnosed conditions. Patients were not eligible for the study if they (i) had a history of systemic disease (e.g., diabetes mellitus, hypertension, gastric ulcer), bone

conditions or chronic pain, (ii) had a severe/serious illness that required frequent hospitalization, (iii) were current smokers, (iv) were pregnant or breastfeeding, (v) had impaired cognitive or motor function, (vi) had taken anti-inflammatory or analgesic drugs in the previous two weeks or were allergic to NSAIDs, or (vii) were unable to return for the Day 4 evaluation. Patients who developed a postoperative infection were to be dropped from the study.

5.3.1.6 Clinical procedures and experimental interventions

For each surgery, a mucoperiosteal flap was lifted under local anesthesia (2% lidocaine with 1:100,000 epinephrine). A no. 701 surgical bur was used to perform an osteotomy. The surgical site was inspected after extraction, and any sharp bone was filed to prevent discomfort. Copious irrigation was applied, followed by closure using 3/0 polyglactin 910 (Vicryl) sutures. Tooth location (right or left), the volume of local anesthesia administered (number of anesthetic tubes used), the time of the surgery (morning only), and the time needed to perform the surgical procedure were recorded. Each participant received an appropriate dose of ibuprofen 400 mg for three days postoperatively. Group 1 received two doses of ibuprofen before the afternoon, as well as a third dose consisting of a placebo (a sugar tablet of the same colour and shape as the active drug tablet) at bedtime; group 2 received three doses of ibuprofen 400 mg three times per day. The oral medications (ibuprofen and the placebo) were identical in appearance, and an investigator gave each patient the medications in a closed envelope labelled with the patient's assigned study identification number, known only by the investigator (Figure 5-12).

Figure 5-12: Diagram of the study flow



5.3.2 Blinding and randomization

In the interest of minimizing bias, both the patient and surgeon were blinded to treatment group allocation. After undergoing a clinical evaluation by the surgeon, patients who met the eligibility criteria were randomized into two experimental groups. One clinician performed the surgical procedure for all the participants. The randomization of participants was completed by an investigator who was not involved in the recruitment or treatment of the participants; the investigator applied permuted-block randomization via the website www.randomization.com. The investigator distributed the ibuprofen and placebo in the appropriate sequence. Other than providing the ibuprofen and the placebo, the investigator had no contact with the study participants and no involvement in data collection or analysis. All the records and test results were identified with codes known only by a person not otherwise involved in the clinical trial.

5.3.3 Study outcome measures

The targeted outcomes were pain relief and healing indicators.

5.3.3.1 Primary outcome measure

For the primary outcome, pain severity in the first three days after surgery, the patient recorded the pain score three times per day using the Verbal Rating Scale (VRS), which has been validated in many clinical trials (223). Patient perception of pain is considered one of the essential health-related quality of life (HRQOL) measures to consider when assessing the healing and success of treatment after surgery, with the recovery of oral function, and the absence of pain and swelling (87, 224, 225). There is much evidence to support the validity, reliability, and ability to detect changes of VRS after oral and other surgeries; the instrument requires little training to administer and score and is acceptable to patients (226). The VRS is also more sensitive than other measures, which has limited categories to select from, unlike visual analogue scale (VAS), for example, which has 101 response levels (0 to 100mm) (227). This scale quantifies the amount of pain that a patient feels, from 0 (no pain) to 10 (the worst pain imaginable).

Patients were instructed to fill out a journal diary (Appendix III) for three days after surgery and deliver the diary to the investigator during the follow-up visit. For ethical reasons, all participants received rescue medication (500 mg acetaminophen) and were instructed to take two tablets as needed, without exceeding 4 g/day. Patients were permitted to take the painkiller whenever they felt considerable pain, per their judgment, and were instructed to wait at least 6 hours between doses and write in the diary each time they used the medication.

All participants were asked to record the types of food they ate for three days after surgery. Additionally, each participant recorded any side effects during those days.

5.3.3.2 Secondary outcome measures

Other target outcomes consist of healing indicators; we used proxy bone-healing indicators, including facial swelling and mouth opening. A single investigator collected these measures at baseline (before the surgical procedure) and on day 4. To assess postoperative swelling, we measured: a) the distance in millimeters from the bottom edge of the earlobe to the midpoint of the chin (symphysis Hirota), called horizontal distance to the symphysis (DHS); b) the distance in millimeters from the bottom edge of the earlobe to the external angle of the mouth, called horizontal distance to the corner (DHC), and c) the distance in millimeters from the palpebral outboard angle to the gonial angle, called vertical distance (DV).

The swelling ratio was calculated as follows: (postoperative measurement − preoperative measurement) × 100/preoperative measurement. Trismus, an inability to open the mouth, may be a consequence of the surgery due to inflammatory processes involving the muscles. We evaluated this condition by measuring the distance between the incisal edges of the upper and lower central incisors with a ruler when the mouth is fully open. Trismus was considered present if the patient had limited jaw opening less than or equal to a 10-mm interincisal distance (223). We also checked and recorded the presence of erythema, muscle tenderness, temporomandibular symptoms and dry socket during the follow-up visit.

5.3.3.3 Explanatory outcome measures

The inflammatory condition associated with wound healing in the two treatment groups was explored thought the expression levels of C reactive protein (CRP) in patients' blood samples. CRP is a protein secreted in the blood plasma, and it is synthesized by the liver as a response to the systemic inflammation that may be caused by different acute or chronic inflammation among

them acute phase response after surgery or trauma such as third molar surgical extraction. In a healthy individual, the CRP level is less than 10 mg/L, increases two folds every 8 hours postoperatively and peak in the third or fourth day after third molar surgical extraction, and keep detected in a higher level for one week (228). Previous studies demonstrate that CRP is an excellent objective indicator for the inflammatory clinical response following third molar surgical extraction and its elevated level was associated with clinical signs of acute inflammation such as facial swelling limited mouth opening (228).

A blood sample (3 ml) was drawn from the cubital fossa of the patients two times in each treatment phase (one hour before the surgical procedure and on day four after surgery). The collected blood samples were centrifuged, and the CRP level in the patient's serum was analyzed in the JUST university lab immediately after collection from the patients and the results recorded using the patient's serial number.

5.4 Statistical analysis

For the in vivo animal studies, all sample sizes calculation for individual experimental groups was based on a coefficient of variation of 25% in the types of data collected, and α and β errors of 5%. The sample size for the groups of the first two experiments was calculated using 0.8 power. All data were expressed as mean ± SE. For all studies in this thesis, the normal distribution of the data was checked using the Shapiro-Wilk test. For comparison between two groups, we used unpaired Student's t-tests when the data were normally distributed; if not, the Mann-Whitney test was performed. We used two-way ANOVA analysis followed by the Fisher LSD post hoc test for comparisons between groups at different time points. Statistical analyses were performed

using OriginPro 2017 (OriginLab, Northampton, MA). Differences were considered significant at $p < 0.05$.

For the cytokines experiment, the sample size of each experimental group was 3 with three replicates; this was selected based on previous studies to achieve an $\alpha=0.05$ and a power of 0.8 according to previous studies (69, 70). For gene expression data, we used the PANTHER classification system (PANTHER overrepresentation test, Reactome Pathways, version 11(2016), www.pantherdb.org) with the whole mouse genome (data updated to NCBI's January 10, 2011 release) (71).

To estimate the sample size for the pilot RCT, we followed the strategy recommended by Whitehead et al. (229) to optimize resources and ensure the precision of estimates of variation in the outcome measures. We require 30 participants in each arm to estimate a standardized difference of 0.25 in pain scores between the intervention and control arms with a power of 80% and Type I error rate of 5%. Therefore, a total of 70 participants were randomized in two arms, taking into consideration a 10% loss to follow-up.

A preface to the manuscript I

Most of the available evidence regarding the effect of NSAIDs administration on fracture bone healing came from preclinical animal studies. However, to best of our knowledge there is no systematic review and meta-analysis available that synthesized the effect of NSAID on specific healing outcomes such as biomechanical properties and histomorphometry analysis. Manuscript I addresses this gap by providing a comprehensive systematic review with meta-analysis of animal studies examining the effect of NSAID administration after fracture surgery on several healing outcomes using different animal models. In addition to providing comprehensive evidence on the subject, this work will also help to design of future animal studies. Bibliography of this manuscript is incorporated at the end of the chapter.

6 Manuscript I

Title: Non-Steroidal Anti-Inflammatory Drugs and Bone Healing in Animal Models – A Systematic Review and Meta-analysis

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Submitted to *Injury Journal* in **April 20, 2019**

Abstract

Background: Non-steroidal anti-inflammatory drugs (NSAID) have excellent anti-inflammatory and analgesic properties and are extensively used to treat post-traumatic or surgical musculoskeletal pain. Although an extensive literature exists on the administration of NSAID on animal bone healing, no systematic review and meta-analysis have yet been conducted to on the subject. Such work is important as it can identify the key histomorphometric and biomechanics characteristics during the process of fracture healing and provide comparative information regarding different factors that may affect this process after NSAID administration.

We performed a systematic review and meta-analysis of animal studies to estimate the effect of NSAID administration after bone fracture on healing outcomes.

Methods: We searched eight databases without limiting the search to starting date up to August 1, 2017 for articles on fractured bone healing in animal models in which NSAID were administered. Out of 5,818 articles screened, 45 were included and three common bone healing outcomes were analysed: biomechanical properties (maximum force to break, stiffness, and work-to-failure), micro-computed tomography (μ-CT), and histomorphometric measurements.

Results: The studies were generally of low-quality scores because crucial information, especially concerning randomization, blinding, and allocation concealment, was poorly reported. There was a significant negative effect of NSAID in rats followed by rabbits regarding mechanical bending healing outcome. Our results show that the negative effects of NSAID after bone fracture on certain biomechanical properties of the healing bones was not statistically significant in mice compared with other animals, in females compared with males, and in younger compared with older animals.

Conclusion: The findings suggest that NSAID should be administered with caution in patients with bone fractures or in those who undergo certain orthopedic surgical procedures until prospective human clinical studies can be conducted.

Keywords: fracture, NSAID, cyclooxygenase, prostaglandin, bone, systematic review and meta-analysis.

Introduction

Non-steroidal anti-inflammatory drugs (NSAID) have been extensively used to treat post-traumatic and surgical musculoskeletal pain because of the excellent anti-inflammatory and analgesic properties of cyclooxygenase (COX) inhibitors (1-5). However, clinical and biological data suggest that COX inhibition has a negative impact on bone tissue repair. Results from several animal trials have suggested that decreased bone healing, including biomechanical and histomorphometric properties, was associated with NSAID administration after bone fracture (6-12). While few studies have investigated the effect of NSAIDs on bone healing in humans, their results are contradictory, possibly reflecting methodological limitations (13-16). Indeed, authors have argued for well-designed, large, multicentric randomized controlled trials with appropriately defined endpoints (17). To the best of our knowledge, there are no clinical studies to accurately determine the effect of NSAID on bone healing.

Several animal models including different animal strains, sex, and fracture techniques have been used to test the effect of types and administration durations of NSAID on bone healing outcomes (10, 18-24). The assessments of bone healing in these studies include integrated multilevel measurements at the organ (e.g., biomechanical to tissue levels using micro-computed tomography (μ-CT) analysis), cellular (e.g., histology, histomorphometric analysis), and gene (e.g., mRNA microarray to explain the associated healing pathway) levels (25, 26). Overall, the techniques used to assess bone healing can be divided into four main categories: imaging analyses, biomechanical tests, detection of serologic markers, and clinical examinations (25). Among these categories, the biomechanical confirmed by histomorphometric analysis is the best way to determine the success of fracture healing (27, 28). These tests are widely used in animal

studies at different time points of fracture healing, which are selected according to the biological, physiological and pathological changes of the fractured bone (28-31).

Although an extensive literature exists on the administration of NSAID on animal bone healing, no systematic review and meta-analysis have yet been conducted on the subject. Such work is important because it can identify the key histomorphometric and biomechanics characteristics during the healing process as well as it provides information on different factors that may affect the healing process after NSAID administration (e.g., NSAID type, animal type and strain, sex of the animal, fracture type, time of assessment). This information may help to design future experimental trials and facilitate knowledge translation. Therefore, we performed a systematic review and meta-analysis of all identified and available animal studies on the effect of NSAID administration after bone fracture on healing outcomes.

Specifically, our objectives were to estimate the extent to which the effect of NSAID administration after bone fracture using animal models: (i) results in less favourable bone-biomechanical and morphometric healing measurements; and (ii) differs by animal type, age and sex, type of NSAID, and length of follow up.

Methodology

The systematic review methodology was specified and documented in advance using SYstematic Review Center for Laboratory animal Experimentation (SYRCLE)'s protocol template for animal studies and registered in the SYRCLE database (Appendix I- Table 12-1S) (32, 33). In our initial protocol, we had proposed to carry out a sensitivity analysis to assess whether the methodological quality of the studies included in the meta-analysis greatly influences the findings

of the review. However, most of the studies in our systematic reviewed received low-quality scores, we therefore amended the review protocol to remove this part of the analysis.

Search strategy and selection of studies

We searched eight databases (Embase, Scopus, Medline, CINAHL, BIOSIS, Cochrane, Central, and DARE) for original articles concerning the effects of NSAID on fractured bone healing in animal models. We considered the period from the starting date of each database to August 1, 2017. The main terms used in the search strategy, developed with the help of the Liaison Librarian for Life Sciences at McGill University, were “anti-inflammatory agents” “non-steroidal”, “bone”, and “animals” (the complete search strategy is provided in Appendix I- Table 12-2S). We used a search filter to detect animal studies and exclude human studies. Although we did not impose language restrictions while searching, only English articles were reviewed. Also, we did not include conference abstracts because they do not provide sufficient data to allow for an evaluation. No other restrictions were used. Additionally, we hand searched the reference lists of eligible articles, and screened review articles for relevant references. The first selection was performed based on independent selection by two reviewers (HA, AP) using RAYYAN software (httpp://rayyan.org, Doha, State of Qatar) (34). Any disagreement was solved by discussion, or by including the third reviewer (BN). Full-text articles were screened and included when they met the following pre-specified criteria: 1) a controlled NSAID interventional design after bone fracture; and 2) a description of outcome measures related to bone healing (biomechanical characteristics, μ-CT scan measures, radiographic bone assessment, and/or histomorphometric and histological based grading). Papers were excluded if they fulfilled one of the following criteria: not an original article (e.g., review or letter), use of a bone graft or other material,

included only outcomes that were not biomechanical or histomorphometric, and duplicate studies. A list of articles excluded as well as the reasons for exclusion are available from the authors upon request.

Data extraction

The final set of articles was assessed independently by two reviewers who extracted the data using DistillerSR (Evidence Partners, Ottawa, Canada) following a piloted data extraction method. Disagreements were resolved by consensus.

Data retrieved from the articles included characteristics of the animals (e.g., animal model used, weight and sex), test methods (bone fracture [e.g., site, type, number and technique used to perform the fracture, whether there was fixation or not, and type of fixation method]), use of medication (e.g., opioid, antibiotics, and NSAID [e.g., type, name, duration of use and route of administration]), and outcome data (time points of outcome measures collection, type of outcome). We grouped the outcomes into main classes: (i)*Biomechanical* (e.g., maximum force or ultimate force load, stiffness, work-to-failure), (ii) μ-CT assessment of healing (volume and density), and (iii) *Histomorphometric characterization* of fracture callus (bone, cartilage, and mineralized tissue).

Raw data or group averages (mean, median), standard deviation (SD), standard error (SE), or ranges and number of animals per group (n) were extracted for all continuous outcome measures. We contacted the authors to obtain original data if results were presented graphically or were incomplete. If data could not be retrieved, the study was excluded from further analysis.

Risk of bias assessment

Two blinded reviewers (HA, AM) assessed the internal validity of the included studies using SYRCLE's risk of bias tool (35). The tool, an adaptation of the Cochrane risk-of-bias tool, considers aspects of bias specific to animal studies. It contains ten entries related to six types of bias (selection, performance, detection, attrition, reporting, and other bias). The score (yes) indicates a low risk of bias, (no) indicates a high risk of bias, and (?) indicates an unclear risk of bias. We were concerned that many items would be rated as having an unclear risk of bias because of the known poor reporting of experimental designs (36). To overcome this problem, we added four entries to the tool, pertaining to randomization, blinding, sample size calculation, and time of day of the NSAID administration or time of day at which surgery was performed (35). For these items, 'yes' and 'no' indicates reported and unreported, respectively.

Data analysis and synthesis

We performed meta-analysis according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines, and using Comprehensive Meta-Analysis software (version 2.2.064, Biostat Inc., Englewood) when five or more independent comparisons from at least three different studies per outcome category were included (provided that outcome measure assessments were sufficiently comparable). We calculated the standardized mean differences (SMD) through Hedges g effect sizes (37). The calculation was (SMD= the mean of the NSAIDs group minus the mean of the control vehicle group divided by the pooled standard deviations of the two groups) to account for the differences in the units of measurements. We used Hedges g effect to calculate the SMD, Hedge's g (which is based on Cohen's D but includes a correction

factor for small sample size bias) (38),(39). Again, the calculations need to take into account the direction of effect.

Despite the anticipated heterogeneity, the individual effect sizes were subsequently pooled to obtain an overall SMD and 95% confidence interval (95%CI) (38, 40). We used a random-effects model (40), which takes into account the precision of individual studies and the variation among them, and weights each study accordingly. If multiple independent experimental groups were compared to the same control group within the meta-analysis, the number of animals in the control group was corrected by dividing it by the number of experimental groups.

Rather than computing a single summary measure, an important objective of meta-analysis is to explore the sources of heterogeneity (41), a measure of the degree of variability in study results, and assess which variables influence the effect of NSAID on bone healing outcomes. We conducted subgroup analyses according to sex and animal species (mice, rats, and rabbits), type of NSAID (non-selective/COX-2 selective), type of fracture, and period of outcome measurement or data collection (early healing less than 21 days, 21 to 48 days, and more than 48 days). We present below the results for subgroups containing at least ten comparisons. A minimum of three independent comparisons per subgroup was needed to record the subgroup characteristics. The interpretation of differences between subgroups should be used mainly to construct new hypotheses rather than drawing definite conclusions. Heterogeneity for subgroup analyses was assessed using I^2 and the Q statistic.

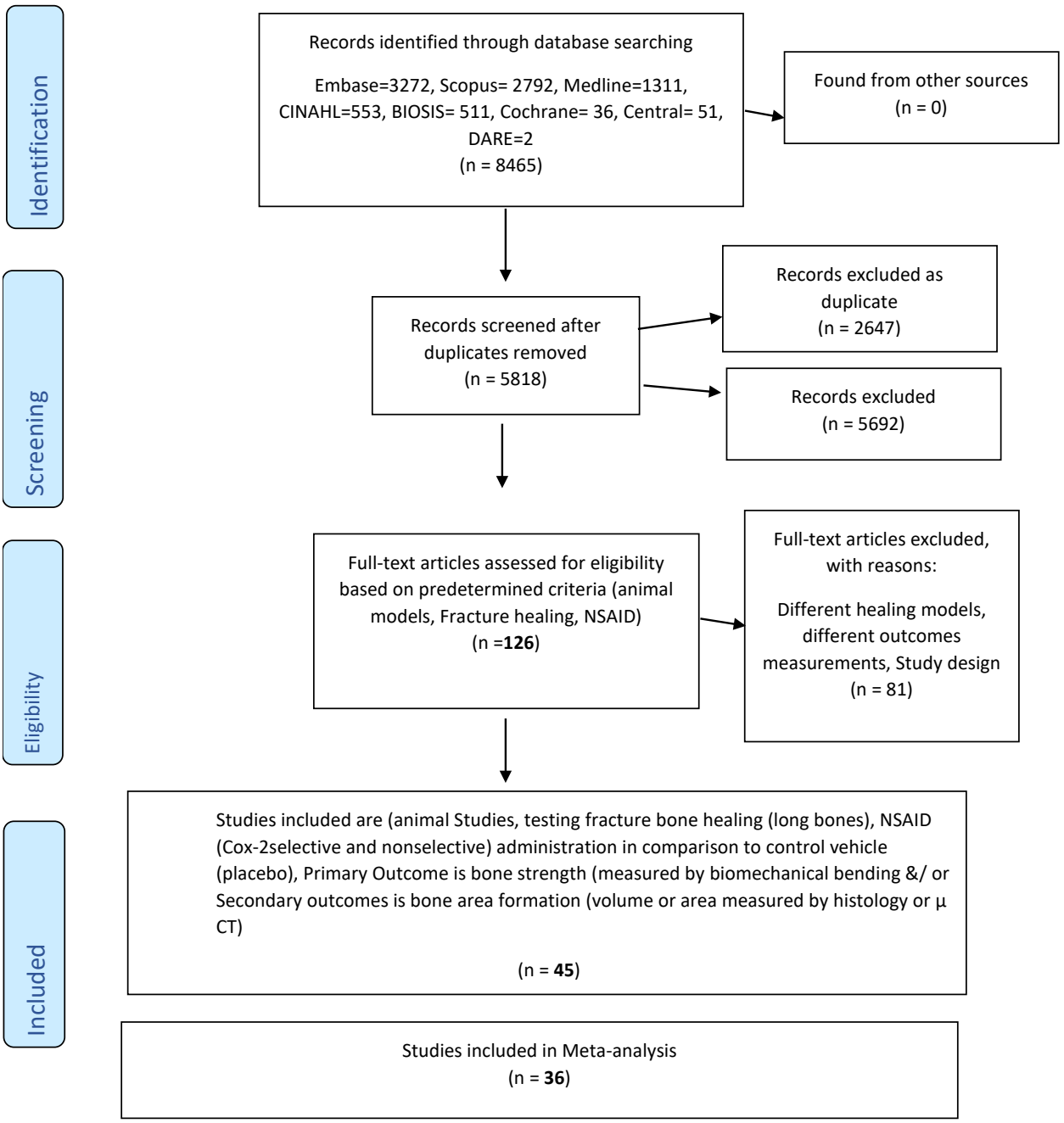
We assessed publication bias for different healing outcomes by evaluating the possible asymmetry using funnel plots and “trim and fill” if the analysis contained at least 20 comparisons.” (38) .

Results

Study selection and characteristics

After the full-text assessment, 45 publications were included in the systematic review (see Figure 6-1 for the PRISMA flowchart). The authors were contacted when we could not retrieve or understand the data, and only two out of ten responded to the request and sent the raw data. (Appendix I- Table 12-3S) presents characteristics of the included studies (the complete list is available in supplementary Excel file on request). Overall, study characteristics varied considerably; most studies were performed in rats (31 studies; 69%), four in mice (8%), nine in rabbits (20%), and one in dogs. Eight studies did not report the sex of the animal, while 24 (53%) and 13 (28.8%) used only male or female animals respectively, and none used both sexes. Eleven studies (24.4%) used a selective COX-2 NSAID as an experimental intervention drug, 22 (48.8%) and 12 (26.6%) used a non-selective or both NSAID types, respectively. There was a great variability on the primary outcomes for biomechanical characteristics; 35 (77.7%) studies reported one or more biomechanical characteristics, and ten (22.2%) studies did not report any of these characteristics.

Figure 6-1: PRISMA flow diagram of the selection of the included studies for the systematic review and meta-analysis



Risk bias and quality of the studies

The assessment results for risk of bias and quality of reporting related to randomization, blinding, sample size calculation, and time of day for NSAID administration or surgery are summarized in Figure 6-2 and Figure 6-3 (The complete risk of bias assessment is available in supplementary Excel file on request). Among the 45 included studies, 28 (62.2%) mentioned the term "randomization" at any step in the study, but no article provided details on the method used. Only 12 (26.6%) studies reported blinding which for most of them was on the histological outcome assessment. Among all included studies, only five (13.3%) reported a sample size calculation; no article specified the time of day at which NSAID was administered or the time that surgery was performed (day or night). Due to poor reporting, many items evaluating the risk of bias on the assessment tool showed an unclear score. For example, "selective outcome reporting bias" was assessed as unclear for all studies because none reported using a research protocol defining primary and secondary outcomes.

Figure 6-2: Risk of bias assessment using SYRCLE risk of bias tool

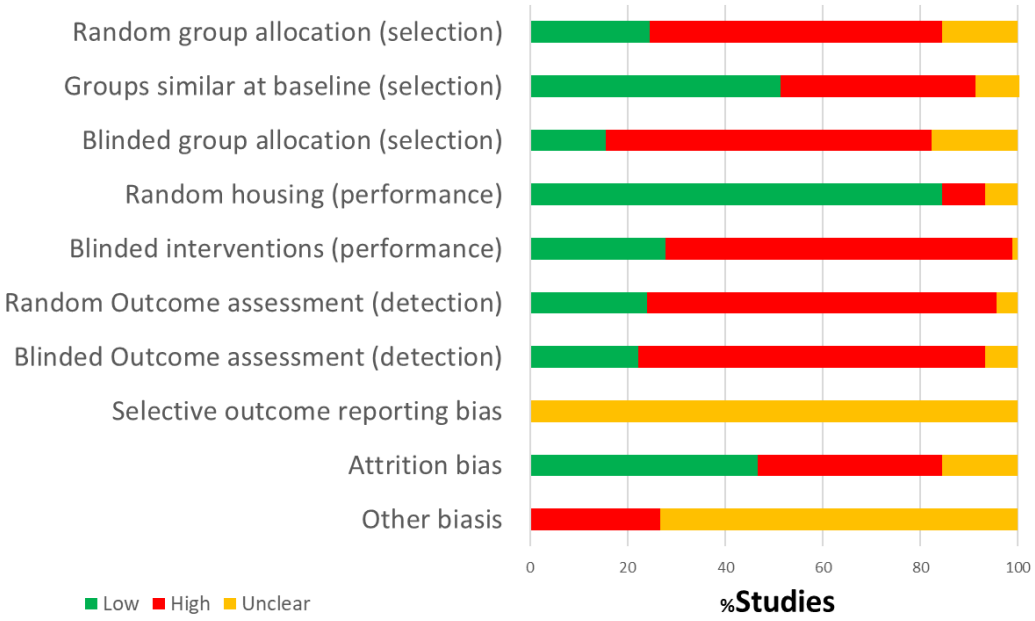
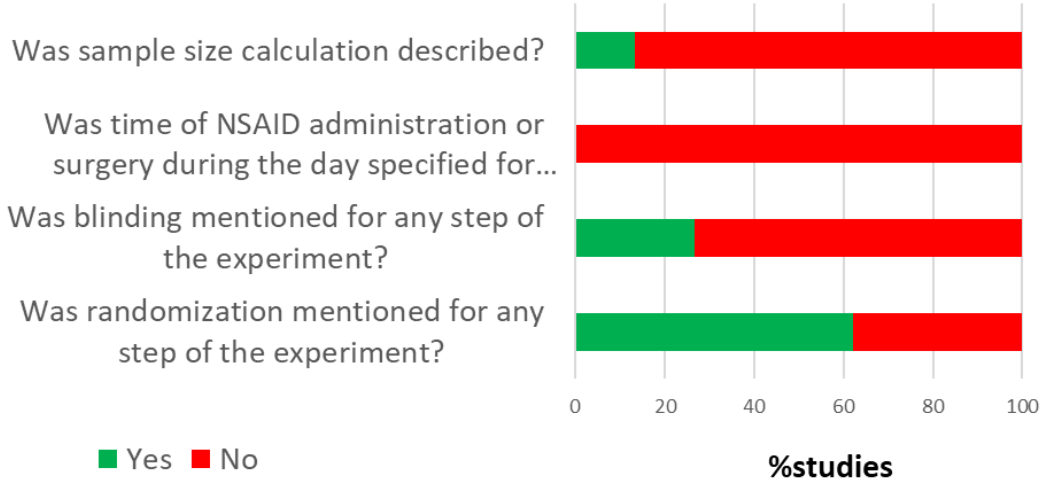


Figure 6-3: Quality of reporting assessment of the included studies



Meta-analysis of NSAID administration during fractured bone healing

Out of 45 studies; we included 36 studies and the reasons for excluding these nine studies were missing information regarding outcome data , lack of measurement of a suitable healing outcome, or the intervention was not a bone fracture (6, 42-49), Thirty-two studies out of the 36 compared the effect of administration of one or more NSAID on biomechanical characteristics (e.g., maximum force (MF) to fracture, stiffness, and work-to-failure) to a control group. For three-point mechanical bending properties, the analysis includes 186 experiments covering different animal models, NSAID types and measurement time points. Four and seven studies were included in the analysis of the effect of NSAID administration on the μ-CT and histological assessment healing outcomes, respectively. The average timing of data collection after bone fracture to assess the mechanical bending maximum force of healing bones was an average of 29.6 days (minimum, 5 days; maximum, 84 days).

Biomechanical assessment

Results from thirty studies including 94 comparisons showed that the maximum force to fracture was significantly decreased, indicating bone healing delay, in animals that received an NSAID after bone fracture compared to the control group (SMD -0.58, 95%CI [-0.74,-0.42]; Table 6-1). Heterogeneity was moderate (I^2, 55.04%). Similarly, animals that received NSAID had an overall decrease in bone stiffness and work-to-failure properties (SMD -0.56 [-0.76,-0.37] and SMD -0.58 [-0.95,-0.20]) respectively compared to controls (Figure 6-4; Table 6-1). Between-study heterogeneity was moderate for both stiffness (I^2, 60.41%) and work to failure outcomes (I^2, 56.29%).

We explored the sources of heterogeneity by examining the effect sizes in predefined subgroups: animal sex, age and species, time of bone collection and type of fractured bone. While animal age and type of bone were source of heterogeneity for the maximum force to break, time of sample collection and animal sex, age, and species were for the stiffness analysis. Moreover, sex and time of sample collection were sources of heterogeneity in the work to failure analysis (Table 6-1).

Table 6-1 shows the subgroup analysis for three-point mechanical bending measurements. For maximum force measurement, NSAID administration did not delay bone healing among mice (SMD -0.28 [-0.68, 0.10]) but did it in other animals. In addition, we observed a difference in this measurement for the subgroup analysis of bone model; while femur (SMD -0.68 [-0.88, -0.48]) showed a significant difference between NSAID and control, tibia did not (SMD -0.19 [-0.49, 0.10]). Moreover, when comparing SMD across bone models, the effect of NSAID administration was significantly larger in femur compared to tibia (P=0.007; Figure 6-5b).

Bone stiffness among mice (SMD -0.07 [-0.55, 0.40]) and animals older than 16 weeks (SMD -0.31 [-0.82, 0.18]) did not differ between NSAID and control groups (Table 6-1). However, compared to controls, bone healing was better in mice taking NSAIDs than in rabbits (p = 0.01; Figure 6-6a). The effect of NSAID administration was significantly different when the bone samples were harvested before 21 days compared to other time points between 21 to 48 days after surgery (P=0.03; Figure 6-6c).

Regarding work to failure, there was no significant effect of NSAID administration on bone healing in the groups of female animals (SMD -0.09 [-0.55, 0.36]), those that received Selective-

cyclooxygenase2 NSAID (SMD, -0.58 [-1.26, 0.09]), and for the femur bone model fracture (SMD, -0.38 [-0.83, 0.05]) (Table 6-1;Figure 6-7).

Table 6-1: Meta-analysis showing the effect of NSAID on mechanical properties after fracture

Maximum Force - NSAID vs Control										
Subgroup	*N*	*SZ NSAIDs*	*SZ Con*	*SMD*	*95% CI*	*P effect*	*Q statistic*	*P within heterog.*	*I^2%*	*P between heterog.*
Overall	94	995	594	-0.58	[-0.74, -0.42]	0.000	206.89	<0.001	55.04	-
Species										
Rats	56	655	370	-0.64	[-0.85, -0.43]	0.000	117.18	<0.001	53.06	
Mice*	17	181	95	-0.28	[-0.68, 0.10]	0.154	54.53	<0.001	70.66	0.336
Rabbits	20	153	124	-0.66	[-1.02, -0.31]	0.000	32.9	0.025	42.26	
Dogs	1	-	-	NA	NA	NA	-	-	NA	
Sex										
Female	22	261	148	-0.61	[-0,95, -0.28]	0.000	62.93	<0.001	66.63	
Male	63	667	377	-0.52	[-0.72, -0.32]	0.000	129.09	<0.001	51.97	0.45
N/M	9	67	70	-0.87	[-1.38, -0.35]	0.000	12.34	0.136	35.2	
Age/ weeks										
<8	3	24	21	0.26	[-0.56, 1.09]	0.536	0.249	0.883	0	
8-16	39	471	270	-0.40	[-0.64, -0.16]	0.001	93.64	<0.001	59.41	0.023*
>16	14	120	63	-0.66	[-1.10, -0.21]	0.003	14.514	0.339	10.43	
N/M	38	380	241	-0.81	[-1.05, -0.56]	0.000	80.64	<0.001	54.12	
Type of NSAIDs										
NS-COX	53	528	349	-0.55	[-0.76, -0.34]	0.000	122.32	<0.001	57.48	0.695
S-COX2	41	467	245	-0.61	[-0.86, -0.37]	0.000	83.79	<0.001	52.26	
Time point										
<21 days	20	214	123	-0.62	[-0.97, -0.27]	0.001	51.81	<0.001	63.33	
21-48 days	58	628	343	-0.54	[-0.75, -0.33]	0.000	124.99	<0.001	54.39	0.957
>48 days	15	129	104	-0.65	[-1.07, -0.24]	0.002	29.25	0.01	52.15	
N/M	1	-	-	NA	NA	NA	-	-	NA	
Type of fractured bone										
Femur	57	662	409	-0.68	[-0.88, -0.48]	0.000	120.03	<0.001	53.34	
Tibia*	28	264	133	-0.19	[-0.49, 0.10]	0.2	62.49	<0.001	56.79	0.01*
Fibula	4	27	11	-0.69	[-1.55, 0.16]	0.115	5.67	0.128	47.15	
Ulna	5	42	42	-1.20	[-1.86, -0.55]	0.000	1.03	0.906	0	
Stiffness - NSAID vs Control										

Overall	76	809	441	-0.56	[-0.76, -0.37]	0.000	189.46	<0.001	60.41	-	
Species											
Rats	50	582	318	-0.57	[-0.80, -0.33]	0.000	109.04	<0.001	55.06		0.046*
Mice *	14	145	59	-0.07	[-0.55, 0.40]	0.758	55.63	<0.001	76.63		
Rabbits	11	76	58	-1.06	[-1.58, -0.53]	0.000	11.14	0.34	10.3		
Dogs	1	-	-	NA	NA	NA	-	-	NA		
Sex											
Female	18	223	124	-0.82	[-1.21, -0.44]	0.000	40.88	0.001	58.41		0.022*
Male	55	568	299	-0.42	[-0.64, -0.19]	0.000	133.42	<0.001	59.52		
N/M	3	18	18	-1.63	[-2.64, -0.62]	0.002	0.78	0.675	0		
Age/ weeks											
<8	3	24	21	0.00	[-0.89, 0.91]	0.98	0	1	0		0.047*
8-16	30	373	193	-0.39	[-0.7, -0.08]	0.01	100.04	<0.001	71.01		
>16*	13	96	36	-0.31	[-0.82, 0.18]	0.21	18.97	0.08	36.75		
N/M	30	316	188	-0.88	[-1.18, -0.58]	0.000	54.75	0.003	47.03		
Type of NSAIDs											
NS-COX	40	411	255	-0.41	[-0.67, -0.15]	0.002	78.83	<0.001	50.52		0.091
S-COX2	36	398	186	0.75	[-1.04, -0.46]	0.000	102.1	<0.001	65.77		
Time point											
<21 days	14	160	84	-0.99	[-1.43, -0.54]	0.000	21.48	0.06	39.49		0.085
21-48 days	51	560	292	-0.42	[-0.66, -0.19]	0.000	144.11	<0.001	65.3		
>48 days	11	89	64	-0.65	[-1.16, -0.13]	0.014	12.11	0.27	17.46		
Type of Fractured bone											
Femur	46	531	299	-0.59	[-0.84, -0.34]	0.000	100.78	<0.001	55.34		0.458
Tibia	23	227	107	-0.39	[-0.76, -0.02]	0.038	78.93	<0.001	72.12		
Fibula	4	27	11	-0.77	[-1.71, 0.16]	0.107	3.95	0.267	24.06		
Ulna	3	24	24	-1.16	[-2.11, 0.20]	0.017	0.32	0.848	0		
Work to Failure - NSAID vs Control											
Overall	16	148	121	-0.58	[-0.95, -0.20]	0.002	34.32	0.003	56.29	-	
Species											
Rats	12	117	92	-0.52	[-0.96, -0.09]	0.017	25.28	0.008	56.49		0.619

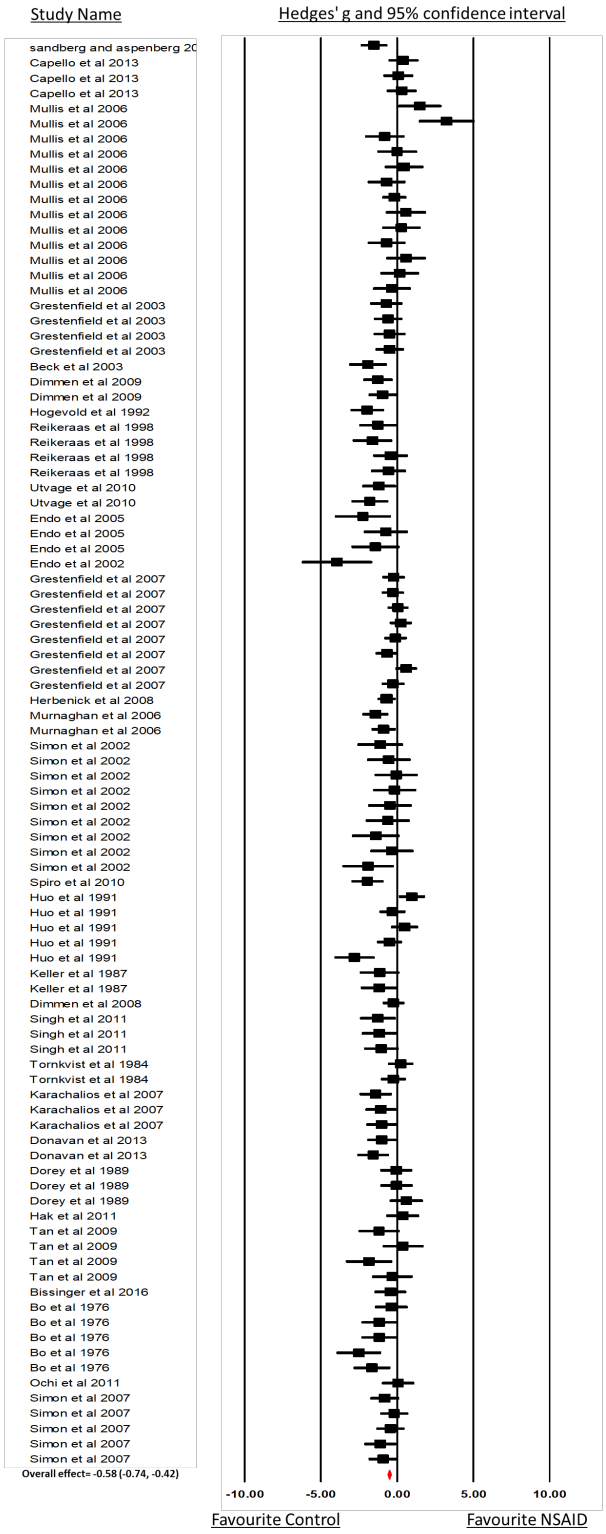
Rabbits	4	31	29	-0.75	[-1.51, 0.01]	0.054	7.72	0.052	61.18	
Sex										
Female*	6	65	66	-0.09	[-0.55, 0.36]	0.694	12.59	0.028	60.29	0.007*
Male	10	83	55	-0.94	[-1.37, -0.52]	0.000	9.63	0.381	6.59	
Age/ weeks										
<8	0	-	-	-	-	-	-	-	-	0.267
8-16	3	24	24	-1.16	[-1.99, -0.32]	0.006	0.32	0.848	0	
>16	3	27	15	-0.67	[-1.54, 0.20]	0.131	7.42	0.024	73.07	
N/M	10	97	82	-0.38	[-0.83, 0.05]	0.089	19.88	0.019	54.73	
Type of NSAIDs										
NS-COX	11	411	255	-0.58	[-1.05, -0.11]	0.015	26.52	0.003	62.30	0.990
S-COX2*	5	398	186	-0.58	[-1.26, 0.09]	0.092	7.72	0.102	48.18	
Time point										
<21 days	1	-	-	NA	NA	NA	-	-	NA	0.030*
21-48 days	12	102	75	-0.71	[-1.10, -0.32]	0.000	19.54	0.052	43.73	
>48 days	3	35	35	-0.59	[-1.26, 0.07]	0.083	2.174	0.33	8.00	
Type of Fractured bone										
Femur*	10	97	82	-0.38	[-0.83, 0.05]	0.089	19.88	0.019	54.73	0.267
Tibia	3	27	15	-0.67	[-1.54, 0.20]	0.131	7.42	0.024	73.07	
Ulna	3	24	24	-1.16	[-1.99, -0.32]	0.006	0.32	0.848	0	

N/M: information not mentioned; N: number of comparisons in analysis; SZ NSAID: number of animals in non-steroidal anti-inflammatory drug group; SZ Con.: number of animals in control group; SMD: standardized means of differences (Hedges' g); CI: confidence interval; NS-COX:, Non-selective cyclooxygenase inhibitor; S-COX2 Selective cyclooxygenase 2 inhibitor; NA: not analyzed because of insufficient data. * need to be explained

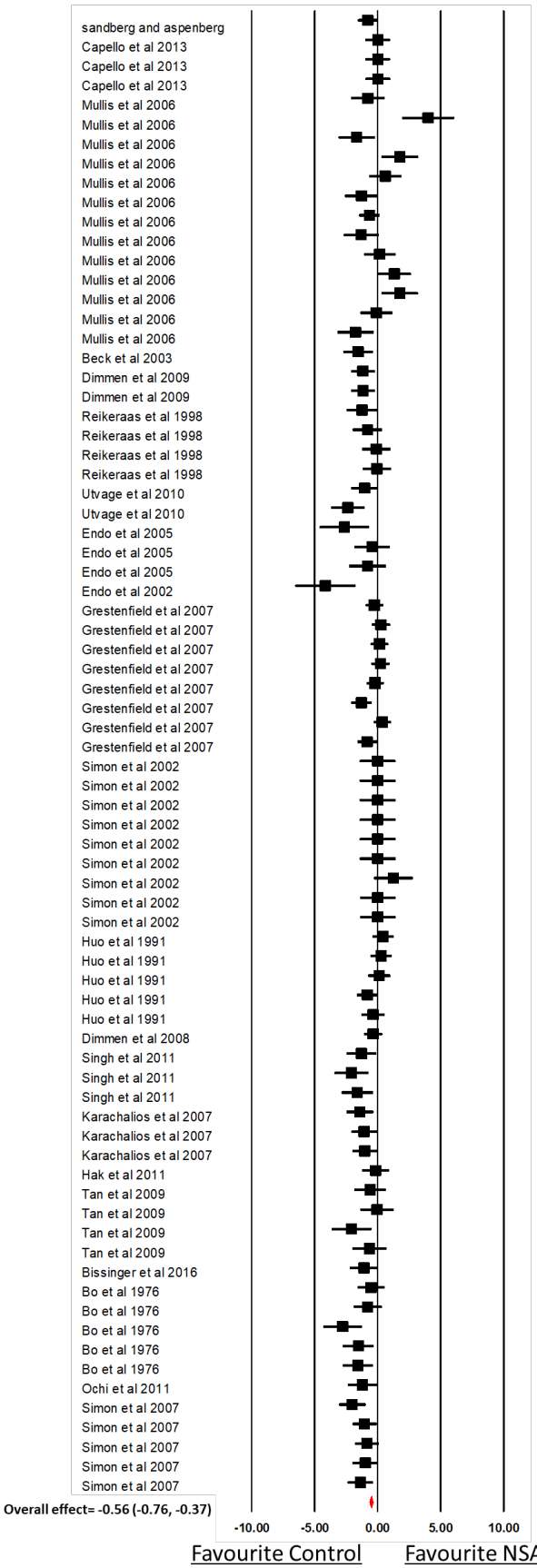
Figure 6-4: Forest plot of the included studies (experimental groups), which used three-points mechanical bending a) maximum force (MF); b) stiffness; c) work to failure.

The forest plot displays the standard mean differences (SMDs) Hedges' g, 95% confidence interval. The diamond indicates the overall estimation and its 95% confidence interval.

a



b


Study Name
Hedges' g and 95% confidence interval
sandberg and aspenberg
Capello et al 2013
Capello et al 2013
Capello et al 2013
Mullis et al 2006
Mullis et al 2006
Mullis et al 2006
Mullis et al 2006
Mullis et al 2006
Mullis et al 2006
Mullis et al 2006
Mullis et al 2006
Mullis et al 2006
Mullis et al 2006
Mullis et al 2006
Mullis et al 2006
Mullis et al 2006
Beck et al 2003
Dimmen et al 2009
Dimmen et al 2009
Reikeraas et al 1998
Reikeraas et al 1998
Reikeraas et al 1998
Reikeraas et al 1998
Utvage et al 2010
Utvage et al 2010
Endo et al 2005
Endo et al 2005
Endo et al 2005
Endo et al 2002
Grestenfield et al 2007
Grestenfield et al 2007
Grestenfield et al 2007
Grestenfield et al 2007
Grestenfield et al 2007
Grestenfield et al 2007
Grestenfield et al 2007
Grestenfield et al 2007
Simon et al 2002
Simon et al 2002
Simon et al 2002
Simon et al 2002
Simon et al 2002
Simon et al 2002
Simon et al 2002
Simon et al 2002
Simon et al 2002
Huo et al 1991
Huo et al 1991
Huo et al 1991
Huo et al 1991
Huo et al 1991
Dimmen et al 2008
Singh et al 2011
Singh et al 2011
Singh et al 2011
Karachalios et al 2007
Karachalios et al 2007
Karachalios et al 2007
Hak et al 2011
Tan et al 2009
Tan et al 2009
Tan et al 2009
Tan et al 2009
Bissinger et al 2016
Bo et al 1976
Bo et al 1976
Bo et al 1976
Bo et al 1976
Bo et al 1976
Ochi et al 2011
Simon et al 2007
Simon et al 2007
Simon et al 2007
Simon et al 2007
Simon et al 2007
Overall effect= -0.56 (-0.76, -0.37)
-10.00
-5.00
0.00
5.00
10.00
Favourite Control
Favourite NSAID

C

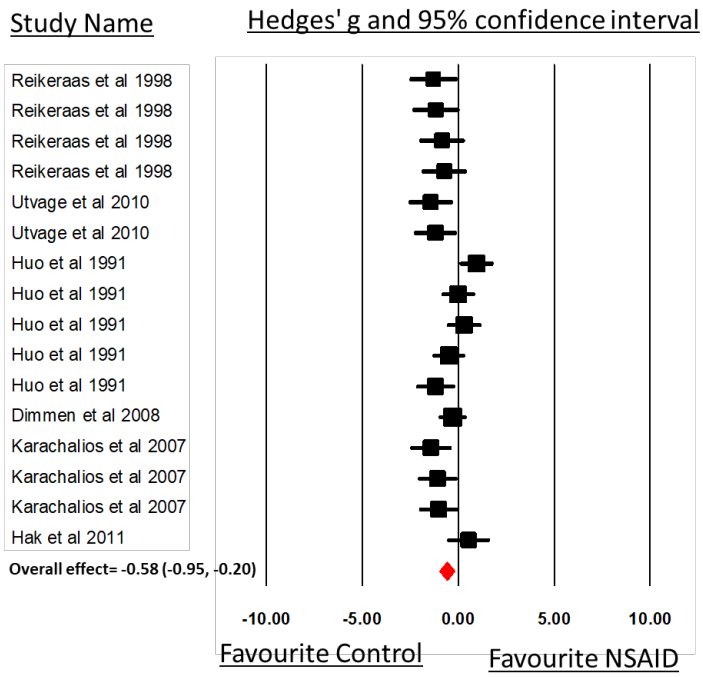

Study Name
Hedges' g and 95% confidence interval
Reikeraas et al 1998
Reikeraas et al 1998
Reikeraas et al 1998
Reikeraas et al 1998
Utvage et al 2010
Utvage et al 2010
Huo et al 1991
Huo et al 1991
Huo et al 1991
Huo et al 1991
Huo et al 1991
Dimmen et al 2008
Karachalios et al 2007
Karachalios et al 2007
Karachalios et al 2007
Hak et al 2011
Overall effect= -0.58 (-0.95, -0.20)
-10.00
-5.00
0.00
5.00
10.00
Favourite Control
Favourite NSAID

Figure 6-5: Effect of (a) Animal models' characteristics; (b) Type of fractured bone; (c) Time of collection healing bones; (d) Type of NSAID; and (e) Age of animals on maximum force to fracture (MF) after administration of NSAID compared to administration of control vehicle.

The columns indicate the effect estimate with the 95% confidence interval of the subgroups. SMD, standard mean difference-Hedges' g mean. * P<0.05 (unpaired student t-test). NM, Not mentioned; NS-COX, Non-selective cyclooxygenase inhibitor; COX2, Selective-cyclooxygenase2 inhibitor.

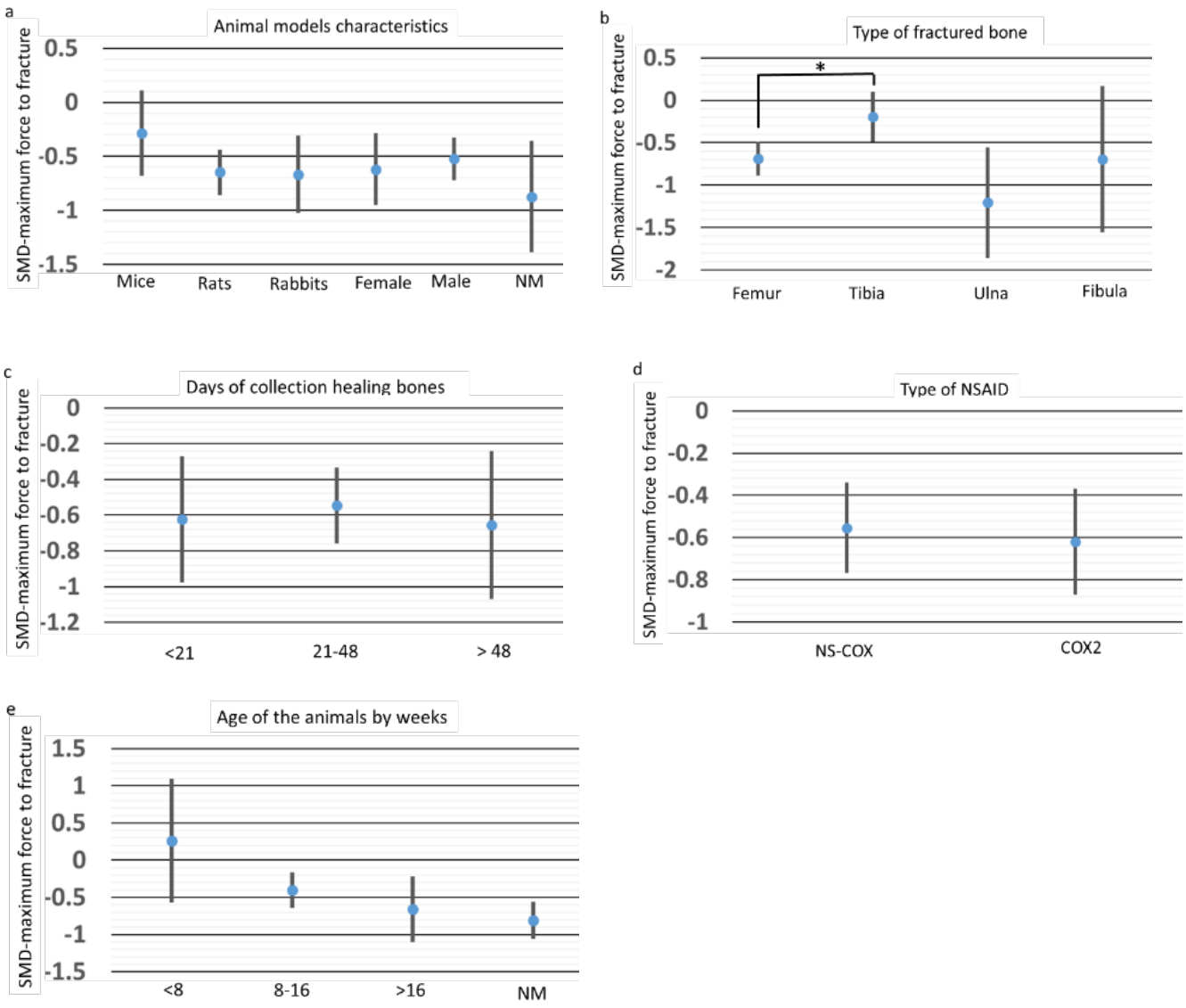


Figure 6-6: Effect of (a) Animal models' characteristics; (b) Type of fractured bone; (c) Time of collection healing bones; (d) Type of NSAID; and (e) Age of animals on (stiffness) after administration of NSAID compared to administration of controlled vehicle.

The columns indicate the effect estimate with the 95% confidence interval of the subgroups. SMD, standard mean difference-Hedges' g mean. * P<0.05 (unpaired student t-test). NM, Not mentioned; NS-COX, Non-selective-cyclooxygenase; COX2, Selective-cyclooxygenase 2 inhibitor.

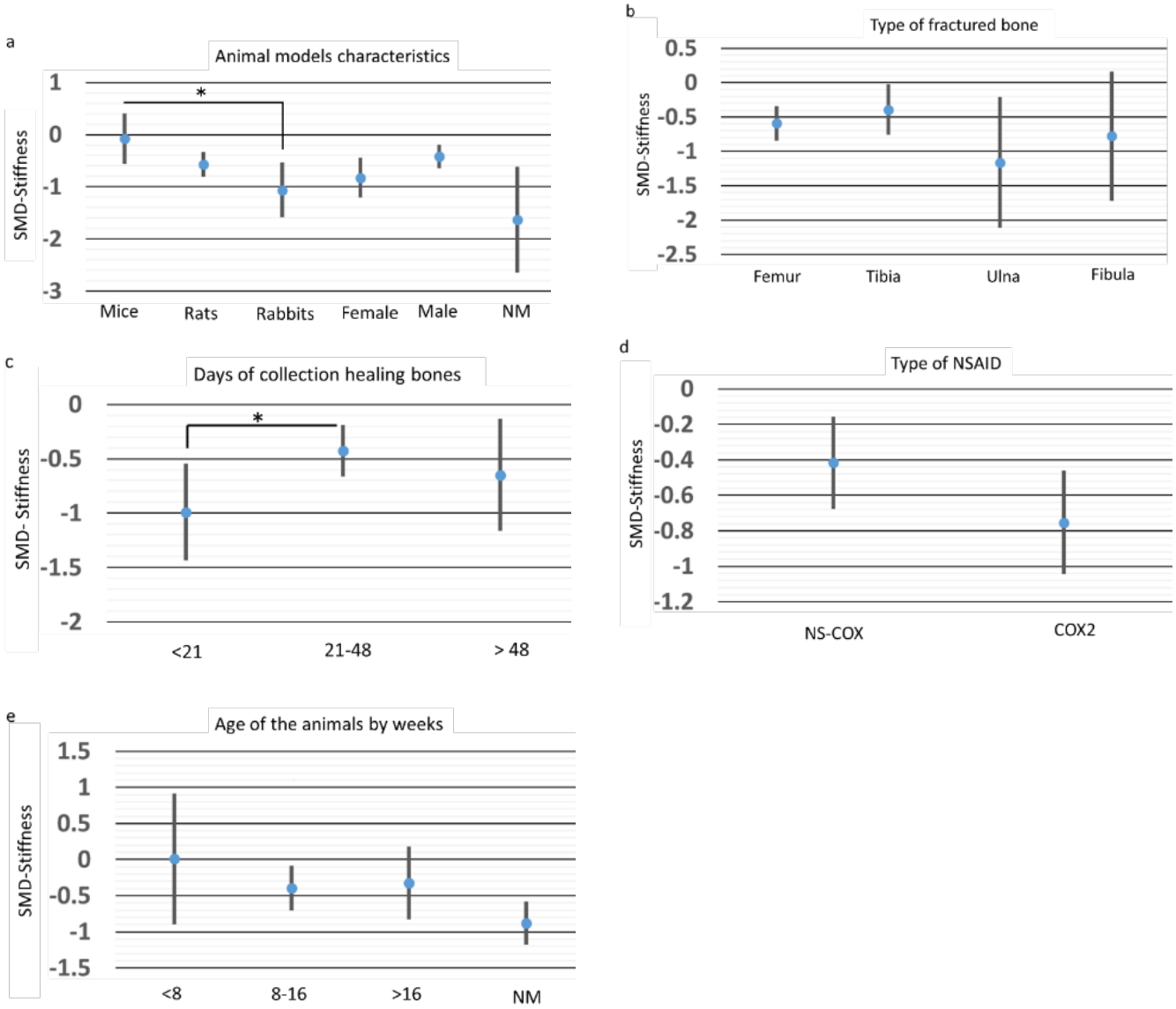
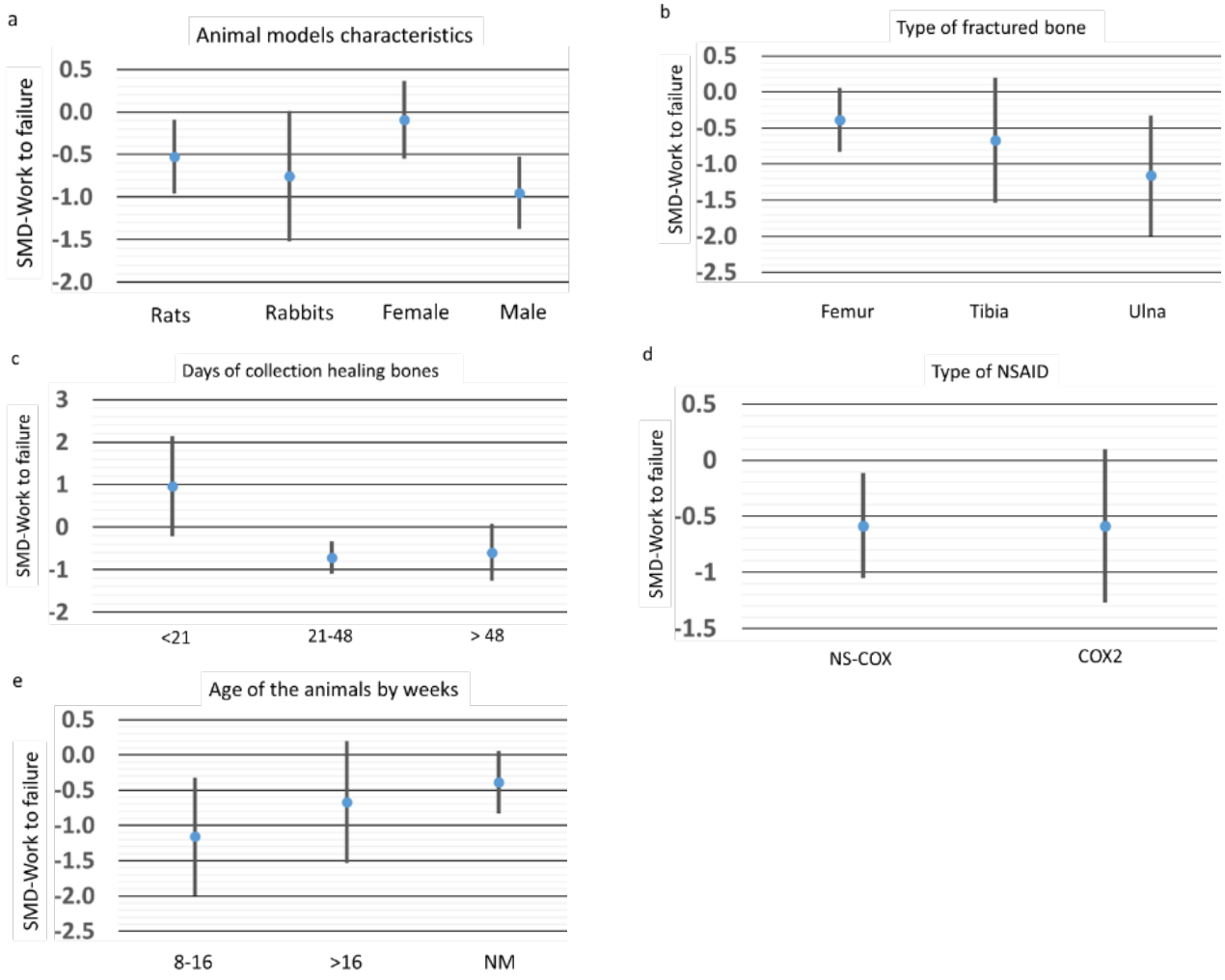


Figure 6-7: Effect of (a) Animal models' characteristics; (b) Type of fractured bone; (c) Time of collection healing bones; (d) Type of NSAID; and (e) Age of animals on (work to failure) after administration of NSAID compared to administration of controlled vehicle.

The columns indicate the effect estimate with the 95% confidence interval of the subgroups. SMD, standard mean difference-Hedges' g mean. NM, Not mentioned; NS-COX, Non-selective-cyclooxygenase; COX2, Selective-cyclooxygenase 2 inhibitor.



µ-CT assessment (bone assessment)

We included five comparisons from four studies that measured healing bone using a µ-CT scan in the meta-analysis. The average time of bone collection after animal euthanasia was 19.5 days (range, 17–21 days). Figure 6-8 and Table 6-2 show the distribution of the data. Although the subgroup analyses were not performed because the number of comparisons was small, the overall analysis shows a significant difference in bone volume measurements for animals that received NSAID compared to controls (SMD, -1.63 [-2.87, -0.39]), but this was associated with high heterogeneity among the studies (I^2 83.32, p <0.001).

Figure 6-8: Forest plot of the included studies (experimental groups), which used µ-CT analysis bone volume and bone density measurements.
The forest plot displays the standard mean differences (SMDs) Hedges' g, 95% confidence interval. The diamond indicates the global (overall) estimation and its 95% confidence interval.

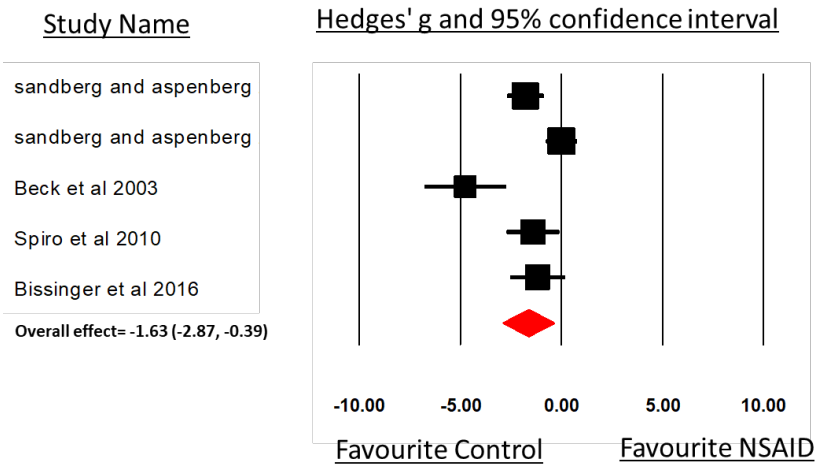


Table 6-2: Meta-analysis based on several subgroups showing the effect of NSAID administration on bone volume and density measurements (µ-CT assessment) of healing bones after fracture.

µ-CT assessment (Bone) NSAID vs Control										
Subgroup	*N*	*SZ NSAIDs*	*SZ Con*	*SMD*	*95% CI*	*P effect*	*Q statistic*	*P within heterog.*	*I^2%*	*Pbetween heterog.*
Overall	5	48	39	-1.63	[-2.87, -0.39]	0.01	23.98	<0.001	83.32	-
Species										
Rats	2	7	3	NA	NA	NA	NA	NA	NA	0.193
Mice	3	31	31	-1.03	[-2.55, 0.48]	0.18	9.95	0.007	0	
Sex										
Female	1	5	5	NA	NA	NA	NA	NA	NA	0.851
Male	4	43	43	-1.73	[-3.06, -0.27]	0.029	23.755	<0.001	87.37	
Age/ weeks										
<8	0	NA	NA	NA	NA	NA	NA	NA	NA	0.007
8-16	4	38	34	-1.04	[-1.98, -0.10]	0.03	10.157	0.017	70.46	
>16	0	NA	NA	NA	NA	NA	NA	NA	NA	
N/M	1	10	5	NA	NA	NA	NA	NA	NA	
Type of NSAIDs										
NS-COX	5	48	39	-1.63	[-2.87, -0.39]	0.01	23.985	<0.001	83.32	1
S-COX2	0	NA	NA	NA	NA	NA	NA	NA	NA	
Time point										
<21 days	4	38	34	-1.04	[-1.98, -0.10]	0.03	10.157	0.017	70.46	NA
21-48 days	1	NA	NA	NA	NA	NA	NA	NA	NA	
>48 days	0	NA	NA	NA	NA	NA	NA	NA	NA	
Type of fractured bone										
Femur	4	38	34	-1.04	[-1.98, -0.10]	0.03	10.157	0.017	70.46	0.007
Tibia	1	NA	NA	NA	NA	NA	NA	NA	NA	

N/M: not mentioned; N: number of comparisons in analysis; SZ NSAID: number of animals in non-steroidal anti-inflammatory drug group; SZ Con: number of animals in control group; SMD: standardized means of differences (Hedges' g); CI: confidence interval; NS-COX: Non-selective cyclooxygenase inhibitor; S-COX2: Selective cyclooxygenase 2 inhibitor; NA: not analyzed because of insufficient data. * need to be explained

Histomorphometric assessment

Seven studies including 33 experimental comparisons between NSAID administration and a control group showed no significant difference in all three (callus size, cartilage, and bone tissue) histomorphometric measurements (SMD, -0.16 [-0.49, 0.17], I^2 = 54.64). Animal models and types of fractured bones were sources of heterogeneity for histomorphometric measurements of healing bones among studies (Figure 6-9; Table 6-3). Interestingly, no mouse model was used to study the histomorphometric measurements related to bone, cartilage, or callus size. Rat models, and histomorphometric evaluation at less than 21 days showed that bone healing was delayed in the NSAID group compared to controls (Table 6-3).

Moreover, when comparing SMD across animal species, bone models, and time of collection, the negative effect of NSAID administration was significantly larger in rats compared to rabbits (P=0.01; Figure 10a), in femur compared to fibula (P=0.02; Figure 6-10b), and in the groups of bone samples that have been harvested less than 21 days (P=0.03; Figure 6-10c) after surgery.

Figure 6-9: Forest plot of the included studies (experimental groups) that used histomorphometric analysis on the microscopic image including callus size, cartilage tissue, bone volume, and bone area measurements.

A forest plot displays the standards mean differences (SMDs) Hedges' g, 95% confidence. The diamond indicates the overall estimation and its 95% confidence interval.

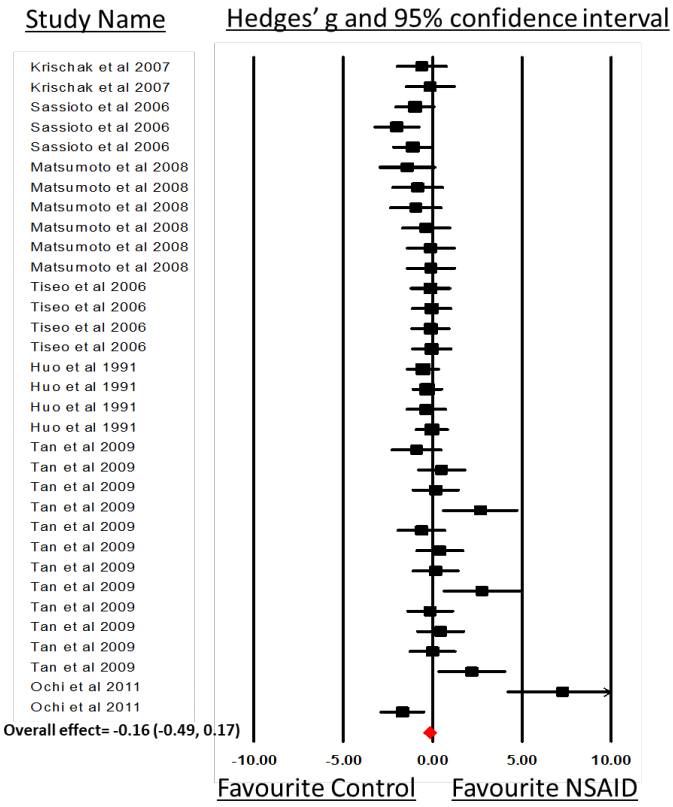


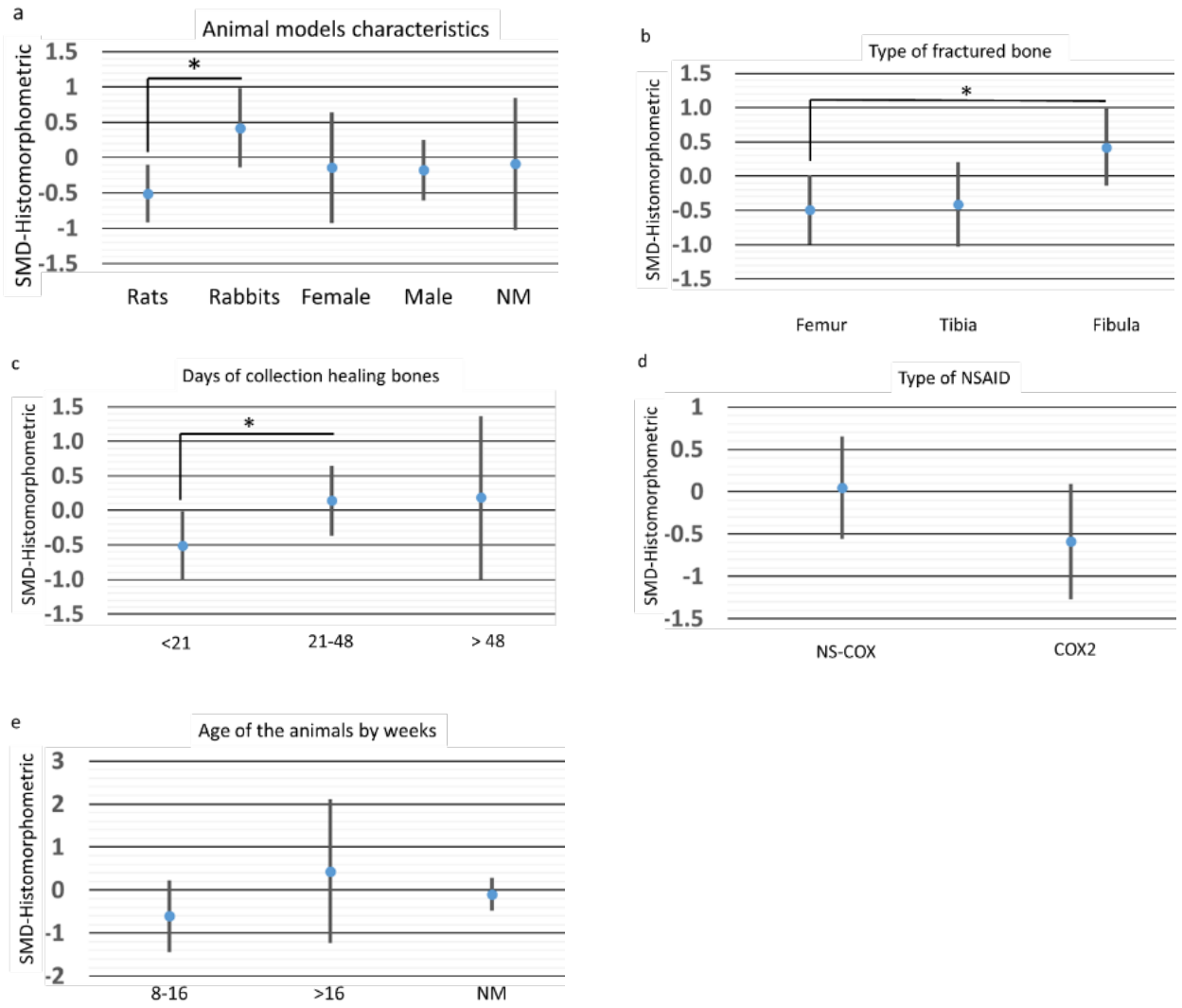
Table 6-3: Meta-analysis based on several subgroups showing the effect of NSAID administration on bone volume measurements (histomorphometric analysis) of healing bones after fracture.

Histomorphometric - NSAID vs Control										
Subgroup	*N*	*SZ NSAIDs*	*SZ Con*	*SMD*	*95% CI*	*P effect*	*Q statistic*	*P within heterog.*	*I^2%*	*P between heterog.*
Overall	33	171	115	-0.16	[-0.49, 0.17]	0.341	70.55	<0.001	54.64	-
Species										
Dogs	2	12	12	NA	NA	NA	NA	NA	NA	0.026*
Rats *	19	123	79	-0.50	[-0.91, -0.10]	0.013	13.26	0.77	0	
Rabbits	12	36	24	0.42	[-0.14, 0.98]	0.143	19.18	0.058	42.65	
Sex										
Female	6	44	43	-0.14	[-0.92, 0.72]	0.723	28.42	<0.001	82.4	0.986
Male	23	106	52	-0.17	[-0.60, 0.25]	0.427	41.66	0.007	47.19	
N/M	4	21	20	-.087	[-1.01, 0.84]	0.854	0.01	1	0	
Age/ weeks										
<8	0	NA	NA	NA	NA	NA	NA	NA	NA	0.426
8-16	6	24	12	-0.60	[-1.43, 0.22]	0.153	2.45	0.783	0	
>16	2	12	12	NA	NA	NA	NA	NA	NA	
N/M	25	135	91	-0.09	[-0.48, 0.29]	0.63	38.63	0.03	37.87	
Type of NSAIDs										
NS-COX	22	130	87	-0.25	[-0.66, 0.14]	0.214	50.72	<0.001	58.6	0.410
S-COX2	11	41	28	0.04	[-0.55, 0.65]	0.878	18.12	0.053	44.81	
Time point										
<21 days*	15	78	46	-0.50	[-1.00, -0.01]	0.042	11.97	0.609	0	0.162
21-48 days	15	73	49	0.14	[-0.36, 0.64]	0.581	25.94	0.026	46.04	
>48 days	3	20	20	0.18	[-0.99, 1,36]	0.758	28.07	<0.001	92.87	
Type of fractured bone										
Femur	11	89	63	-0.49	[-0.99, 0.01]	0.055	10.40	0.406	3.869	0.041*
Tibia	10	46	28	-0.41	[-1.03, 0.20]	0.192	30.63	<0.001	70.61	
Fibula	12	36	24	0.42	[-0.14, 0.98]	0.143	19.18	0.058	42.65	

N/M: information not mentioned; N: number of comparisons in analysis; SZ NSAID: number of animals in non-steroidal anti-inflammatory drug group; SZ Con: number of animals in control group; SMD, standardized means of differences (Hedges' g); CI: confidence interval; NS-COX: Non-selective cyclooxygenase inhibitor; S-COX2: Selective cyclooxygenase 2 inhibitor; NA: not analyzed because of insufficient data. * need to be explained

Figure 6-10: Effect of (a) Animal models' characteristics; (b) type of fractured bone; (c) Time of collection healing bones; (d) Type of NSAID; and (e) Age of the animals on histomorphometric measurements (callus, bone) after administration of NSAID compared to the control vehicle.

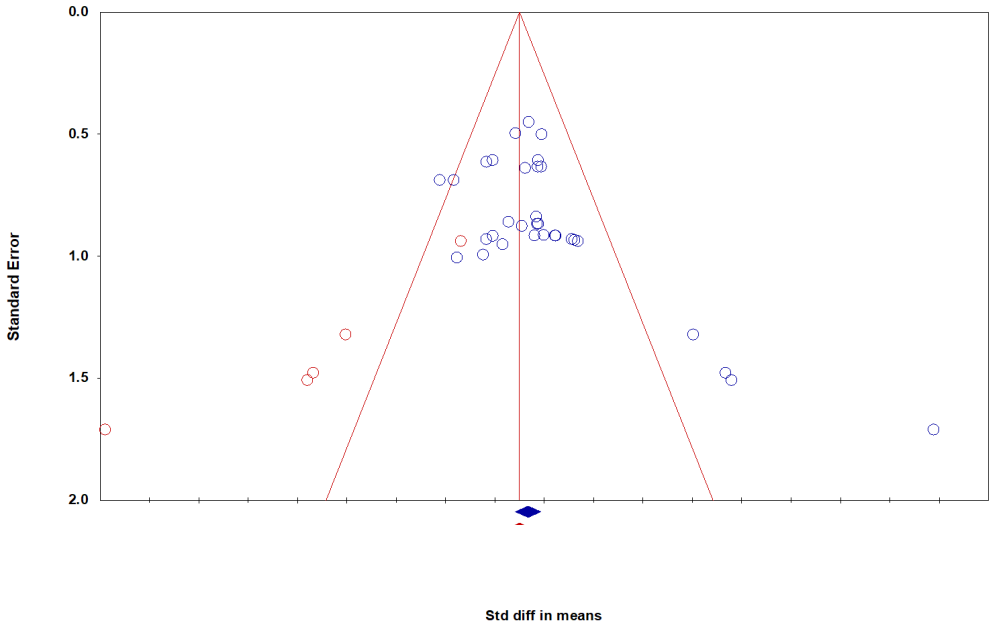
The columns indicate the effect estimate with the 95% confidence interval of the subgroups. SMD, standard mean difference-Hedges' g mean. NM, Not mentioned; NS-COX, Non-selective-cyclooxygenase; COX2, Selective-cyclooxygenase 2 inhibitor.



Publication bias

The possible presence of publication bias was observed when assessing the histomorphometric outcome measurements. The inspection of the funnel plot suggested asymmetry resulting from the underrepresentation of studies that show negative effect of NSAID (Figure 6-11). Trim and fill analysis resulted in five extra data points, indicating the presence of publication bias.

Figure 6-11: Funnel plot for histomorphometric measurements of bone and cartilage in the healing callus



Discussion

This unique systematic review and meta-analysis was designed to answer a specific research question regarding the effect of different types of NSAID on bone fracture healing in animal models. Three important outcomes commonly used to assess bone healing were analyzed: biomechanical properties (maximum force to break, stiffness, and work to failure), micro CT, and histomorphometric measurements.

NSAID administration had a negative effect on the biomechanical properties in different animal models of the included studies (10, 12, 18, 19, 47, 50-58). However, the results for histomorphometric assessments did not show a difference (Table 6-3).

Depending on the type of NSAID, they are known to inhibit both COX isoforms. Both non-selective NSAID and COX-2-selective drugs decrease prostaglandin production, which plays an essential regulatory role in all phases of bone healing, especially the inflammatory phase (59-62). Thus, it is reasonable to expect that NSAID administration after bone fracture may delay or impair healing outcomes (12, 52, 61).

As early as the 1970s, many animal trials strongly emphasized the negative effect of NSAID on bone healing (7, 9, 12, 19, 24, 44, 52, 58, 63). Most studies that used rodents and rabbits demonstrated that non-selective and selective COX inhibitors impair the bone healing process (7, 9-12, 19, 24, 44, 52, 58, 63). Conversely, only a few studies indicated that NSAID has little or no effect on fracture healing outcomes (20, 55, 56, 64). However, these studies have significant limitations, because they tested only one time point, did not measure clear bone healing outcomes that include mechanical or histomorphometric analysis, or used very low NSAID doses, and some did not perform a proper statistical analysis (42, 65, 66). Clinically, there are few retrospective studies and even fewer prospective clinical trials (13-16). The results from the retrospective studies were contradictory. Some of them have confirmed the negative effect of NSAID on bone formation and healing following hip or femoral neck fracture as well as hip arthroplasty (2, 13), while others have shown no effect on bone healing. One of the few prospective clinical trials reported beneficial effects of NSAID on bone healing in humans, which was among Colles' fracture patients who were treated with casting and reduction (14, 16). It is

widely accepted that trying to understand the effect of NSAID administration on bone healing is extremely challenging in a clinical setting especially from a methodological perspective. For example, controlling the many confounding factors (e.g., smoking, diabetes, obesity) in a prospective manner requires considerable time and planning (17). Therefore, it is imperative to translate the evidence from the available in-vivo experimental studies. In fact, animal studies helped in understanding the physiological process of bone healing and can also provide important insights on the effect of NSAID on bone healing.

Some methodological issues that might hamper the interpretation of the experimental animal data and their subsequent translation to the clinical setting should be discussed. First, there was substantial heterogeneity among the various animal studies. We performed subgroup analyses to investigate factors that may modify the effect of NSAID on bone healing outcomes (e.g., animal species, sex, type of bone, age of the animal, type of NSAID, and time at which the outcome was measured. More studies are required, especially in mice, because contrary to other models such as rats, mice show no negative effect of NSAID on the stiffness of the harvested bone compared to the control group. Because the mouse genetic map is similar to that of humans, the mouse may be the best available model to study the effect of NSAID on bone healing in different human genetic conditions (67). Overall, the pharmacokinetic variations between species, sexes, and ages should be considered, especially regarding drug absorption. Our meta-analysis showed a non-significant negative effect of NSAID administration after bone fracture not only in mice compared to other animals, but also in females compared to males, and in younger compared to older animals. Experimental studies that compare different sexes and ages within the same experiment are needed for stratification and comparison.

We observed that the negative effect of NSAID administration on biomechanical properties differs between large (rabbits and rats) and small (mice) species. This suggests that mouse models may be more sensitive than rats for bone healing outcomes, which further supports our argument that they comprise the preferable models for future animal studies. Additionally, this finding suggests that the negative effect of NSAID on bone healing is species-specific for certain outcomes, and this need to be taken into consideration for knowledge translation. The timing of healing outcome measurements also seems to modify the results because they do not show a significant effect of NSAID administration on μ-CT and histomorphometric outcomes early in the healing process compared to the control groups, but results differ when measurements are taken after 21 days. It is important to consider this information during the design of further experimental protocols, and it may be crucial in managing research efforts and reducing the unnecessary use of animals.

Finally, we did not find any studies that used animals of both sexes in their experimental design, which seems to be important for future studies because results differed between males and females for the work-to-failure biomechanical outcome.

Methodological quality of the studies

Our study quality checklist assessed aspects of both internal and external validity, and we observed many studies were generally of low-quality scores and tended to overstate the effect size. The overall quality score accounted for a significant proportion of between-study heterogeneity; however, the correlation between the aggregate quality score and the effect was not clear. The reporting and risk of bias assessments indicate the need for protocol registration or publication, and for the reporting of the elements of randomization, blinding, and allocation

concealment (35). This concern is shared and addressed by others (33, 35, 38). It is crucial that future animal studies improve the reporting of study procedures, allowing others to replicate and build on previously published work. With better reporting, systematic reviews of higher quality will also become feasible.

Clinical implications

Drug pharmacokinetics vary between species, and this must be considered when extrapolating data from animals to humans. Therefore, it is important to investigate NSAID doses that are equivalent to those used in humans after bone fracture (same proportion to animal weight) and to evaluate effects using different types of animal and bone fracture models. The increasing amount of evidence from animal studies and the results from this systematic review and meta-analysis indicate that caution should be exercised when using NSAID after bone fractures or with specific orthopedic surgical procedures until prospective human clinical studies indicate otherwise.

Conclusions

Our findings provide some guidance for future laboratory and clinical research. First, it is important to test different hypotheses of bone fracture healing in small animals, and mice especially because mice models provide opportunities to examine genetics and create knock-out species. Our results also indicate the need for studies that compare the effect of NSAID administration on bone healing outcomes between male and female animal models. Overall, the appropriate animal model to test the effect of NSAID on fracture bone healing should take into consideration the animals' species, type of the bone, and age of the animal.

Moreover, our results demonstrate it is important to choose the suitable time of sample collection based on what healing outcome to be measured. Histomorphometric outcome measurements require more than 21 days to show results that are comparable to the control group. Second, improvements in internal (study quality) and external (publication bias) validity might provide more information for the translation of the data to clinical trials, and more robust exploration of the efficacy limits in such studies could inform inclusion and exclusion criteria for these trials.

It is increasingly clear that the function of COX and their products are critical for bone healing. Most animal and human studies support the conclusion that NSAID administration can delay or impair bone fracture healing. However, the anti-inflammatory and analgesic effect of NSAID seems to be beneficial in treating post-traumatic pain and edema. The need for a new approach to using NSAID that preserves its pain control properties without affecting bone healing is justifiable and important.

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A preface to manuscript II

In the following chapter, I present a series of preclinical experiments that tested the hypothesis that NSAIDs administration during the active phase of the day reduces pain and improves healing recovery after bone fracture surgery. Moreover, this manuscript include experiment to identify inflammatory cytokines and gene expression levels associated with NSAID chronotherapy after three days of fracture bone healing. These studies highlight the importance of this simple treatment approach in controlling pain and promoting healing following bone fracture surgery in mice. Bibliography of this manuscript is incorporated at the end of the chapter

7 Manuscript II

Non-Steroidal Anti-Inflammatory Drugs' (NSAID) Chronotherapy may Enhance postoperative recovery

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To be submitted to *Science Translational Medicine Journal*

One Sentence Summary: Timing of NSAID administration (chronotherapy) after surgery reduce pain and improve postoperative recovery and affect the expression of clock genes.

Abstract

Postoperative pain relief is crucial for full recovery. With the ongoing opioid epidemic and the insufficient effect of acetaminophen on severe pain; non-steroidal anti-inflammatory drugs (NSAID) are heavily used to alleviate this pain. However, NSAID are known to inhibit postoperative healing of connective tissues by inhibiting prostaglandin signaling.

Pain intensity, inflammatory mediators associated with wound healing and the pharmacological action of NSAID vary throughout the day due to the circadian rhythm regulated by the clock genes. According to this rhythm, most of wound healing mediators and connective tissue formation occurs during the resting phase, while pain, inflammation and tissue resorption occur during the active period of the day.

Here we show, in a murine tibia fracture surgical model, that NSAID are most effective in managing postoperative pain, healing and recovery when drug administration is limited to the active phase of the circadian rhythm. Limiting NSAID treatment to the active phase of the circadian rhythm resulted in overexpression of circadian clock genes, such as Period 2 (Per2) at the healing callus, and increased serum levels of anti-inflammatory cytokines interleukin-13 (IL-13), interleukin-4 (IL-4) and vascular endothelial growth factor. By contrast, NSAID administration during the resting phase resulted in severe bone healing impairment.

Key words: Animal models, NSAID, surgery, Healing, Circadian rhythm

Introduction

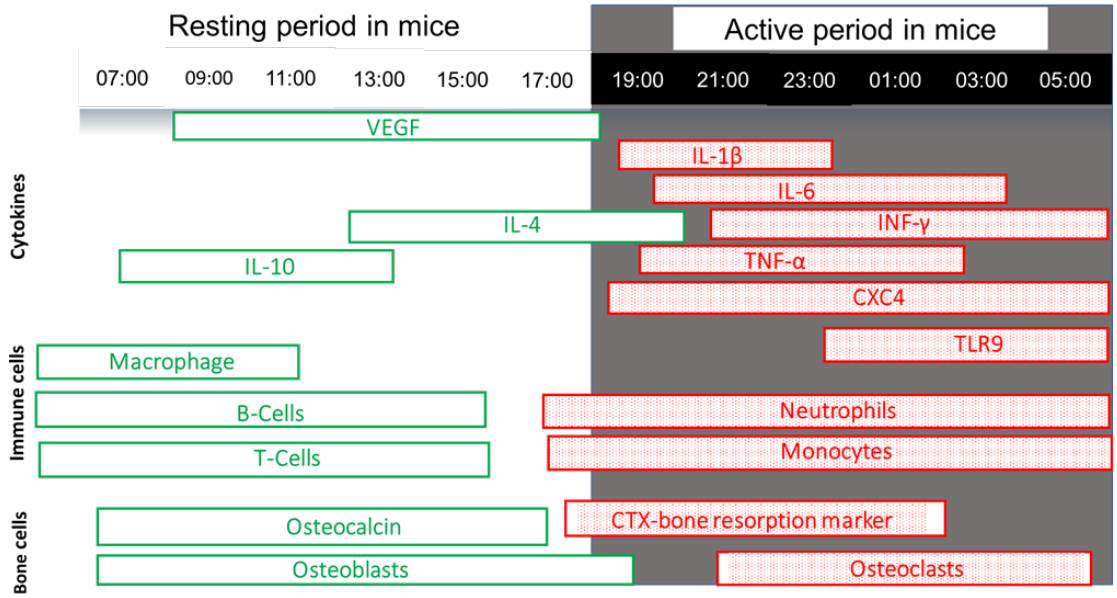
Postoperative recovery following invasive surgical interventions is usually painful and can cause significant morbidity and even mortality in some cases (1). Postoperative pain is a response to injury in which inflammatory mediators are released, inflammatory cells infiltrate the damage site, and nociceptive nerve fibres are activated to produce pain (2). Pain relief is crucial for full recovery; it is essential for the healing process and to resume physical activities (3). However, drugs typically used for postoperative pain management are problematic. Acetaminophen is ineffective in severe pain, and while NSAID and opioids are useful for controlling surgical pain (4), opioids can cause constipation and addiction, and NSAID can delay healing (5-8). Therefore, there is an urgent need for better strategies to manage postoperative pain. One way of achieving this is by developing NSAID treatments that control pain and inhibit inflammatory catabolic activities while sparing the anabolic pathways of wound healing.

Inflammation is essential in healing (9). For example, when connective tissues are injured, an inflammatory response starts by the conversion of arachidonic acid (AA), either into prostaglandin H2 (PGH2) via cyclooxygenase (COX), or into interleukotrien A4 (LTA2) via 5-lipoxygenase (5-LO). Downstream specific synthetase enzymes convert PGH2 and LTA2 into bioactive lipid mediators, such as prostaglandin E2 (PGE2), which play a crucial role in tissue repair (9, 10). Pro-inflammatory cytokines production, along with growth factors activation after injury, results in increased secretion of prostaglandins. Prostaglandins contribute to the regulation of mesenchymal cells differentiation into progenitor osteoblasts (11), thus by inhibiting the production of PGE2 via COX, NSAIDs reduce the production of PGE2, and

subsequently inhibit the healing of connective tissues (bone, cartilage, dermis) (Appendix II-Table 13-1S).

All living organisms possess a circadian rhythm that anticipates the response to light and temperature changes during the 24-hour cycle (12). The circadian system in mammals is composed of a central clock within the suprachiasmatic nuclei and a peripheral clock inside all cells. The circadian clock is controlled through a feedback loop of heterodimer core clock genes composed of circadian locomotor output cycles kaput (Clock) and brain and muscle Arnt such as protein-1 (Bmal1). Clock and Bmal1 drive the expression of two inhibitors, cryptochrome (Cry) and period (Per) (13). This molecular clock modulates the immune response and the healing processes in connective tissues (13) (Figure 7-1 and appendix II-Table 13-2S). For instance, macrophage activity, leukocyte recruitment, and pro-inflammatory mediators such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-12 (IL-12) increase at the beginning of the daily activity. During this phase, the levels of Tol-Like Receptors TLR9 and TLR4 also increase, leading to the upregulation of CCL2, CXCL1, CCL5, and subsequent leukocyte recruitment and potential tissue damage in injured sites (12-15)(Figure 7-1). By contrast, anti-inflammatory mediators and other growth or angiogenesis factors, such as the vascular endothelial growth factor (VEGF), peak during the resting phase (13, 16, 17) (Figure 7-1 and appendix II- Table 13-2S).

Figure 7-1: Diagram of the circadian rhythm in immune cells, pro-inflammatory and anti-inflammatory mediators, bone resorption and formation markers.



The circadian rhythm affects many aspects of connective tissue metabolism (18). A 24-hour oscillation occurs in bone tissue during growth (19), formation, resorption (20, 21), and in the endochondral ossification during bone fracture healing (21). Bone formation occurs during the resting period, and resorption occurs mostly during the active period (21). Experimental studies in rodents and humans reveal that the disruption of sleep and circadian rhythm impairs bone formation (22). All bone cells such as osteoblasts, osteoclasts, and chondrocytes express clock genes, such as Per or Cry, that influence bone volume regulation (23, 24). Cry2 influences the osteoclastic activity and Per2 regulates osteoblast activity (25).

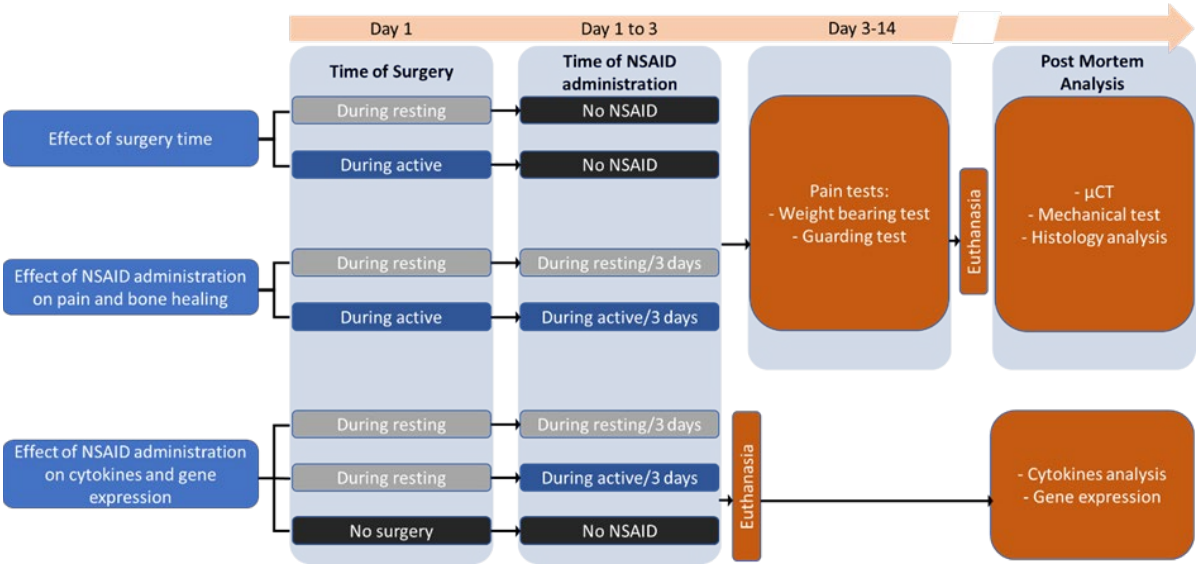
The circadian clock also affects pain, with sensitivity peaking during the active phase (26). Part of the pain response oscillation could be explained by changes in COX-1 and COX-2 activity throughout the day (27), especially after an injury or insult (28). These variations may contribute to the clinically evident circadian variations in the pharmacokinetics effects of NSAID. Specifically,

maximum absorption and effectiveness are achieved when the drug is administered during the active phase when animals are awake (29-33).

Different clinical studies suggest that careful selection of the time of administration can improve the effectiveness of NSAID and can markedly reduce their undesirable effects (34). These drugs exert a strong anti-inflammatory effect when ingested or injected in the morning or early afternoon, but not in the evening, when the risk of its adverse effects such as indigestion, stomach ulcers, and acute kidney problems increase (35).

In this study, we investigated how does the time of NSAID administration impact postoperative pain and healing. We **hypothesized** that circadian variations in inflammation caused by clock genes could determine the postoperative effectiveness of NSAID therapy. Accordingly, limiting NSAID administration to the beginning of the daily activity phase should improve recovery. To test our hypothesis, we assessed the effect of surgery time and time of NSAID delivery on pain and healing outcomes in a bone fracture surgical model in mice. We determined the most efficient delivery time for NSAID after bone fracture surgery. Moreover, we investigated the molecular differences between the healing sites of mice that received NSAID during the active phase and those that received it during the resting phase by characterizing the gene expression profile of fracture calluses in both groups.

Figure 7-2: Study design and objectives



Results

The timing of bone fracture does not affect bone healing and recovery

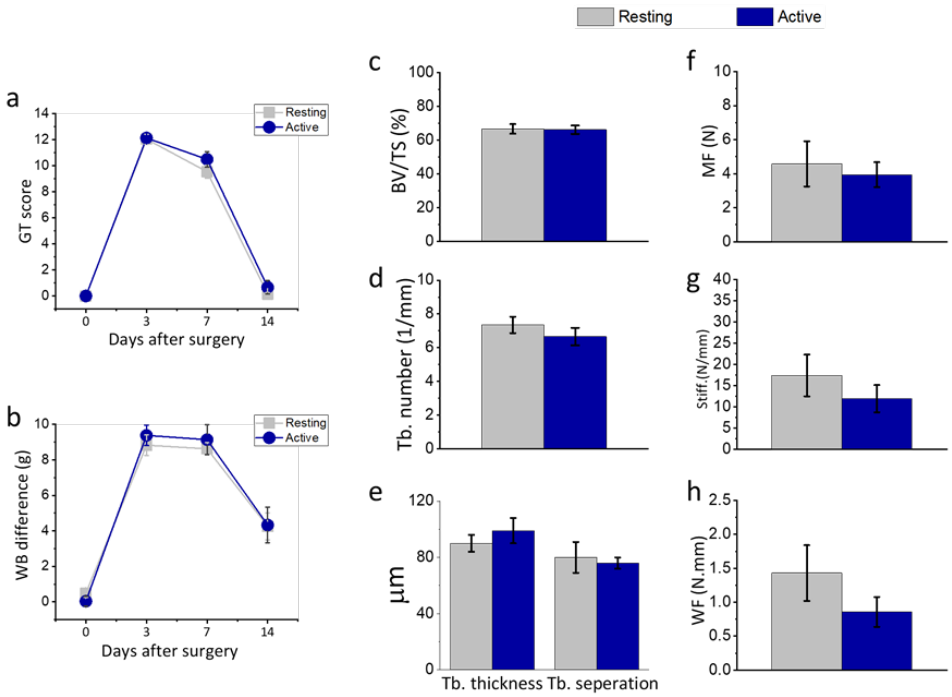
To set the basis for the assessment of NSAID chronotherapy, we first investigated the effect timing of bone fracture on bone healing and recovery. We carried out this experiment by comparing pain behaviour and bone healing of mice that received tibia fracture surgery during the resting phase (when animals are sleeping) to those that received the fracture during the active phase (when they are awake) (Figure 7-2).

In both groups, there were no significant differences in pain behaviours between mice who received tibia surgery during the active or resting phases (Figure 7-3a,b). The guarding of the injured limb increased, but it returned to almost pre-fracture levels by day 14. Similarly, the tendency to bear more weight on the uninjured versus the injured limb increased after the bone fracture, but partially resolved by day 7.

Two weeks following surgery, the osteotomy sites of the fractured tibiae were subjected to μ-CT analyses to assess bone healing (Figure 7-3c,d,e). We did not observe significant differences in the bone volume to tissue volume (BV/TV), callus size, trabecular number (Tb.N), or spacing between the two experimental groups. Moreover, the biomechanical analysis revealed no significant difference in the force to fracture, stiffness or work to failure measurements between the bones harvested from the two experimental groups (Figure 7-3f,g,h).

Figure 7-3: Effect of bone fracture surgery timing on pain and bone healing.

(a,b) Pain assessment, (c,d,e) µCT analysis and (f,g,h) mechanical analysis of tibiae fracture during resting time and active time at day 14 after tibia fracture surgery. The data show that both groups (n=8 per group) had similar pain behaviour (weight bearing (WB), guarding tests (GT)), bone volume fraction (BV/TV), trabecular number (Tb.N), spacing between the trabecula (Tb.Sp), trabecular thickness (Tb.Th), maximum force (MF), stiffness (S) and work to failure (WF). All data are expressed as Mean ± SE values.

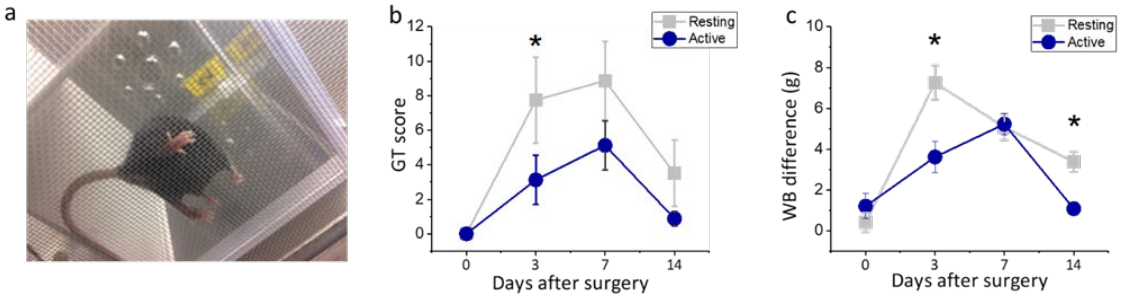


The timing of NSAID administration affects bone fracture pain and recovery

To assess the effect of the timing of NSAID administration on bone fracture pain and recovery, we used a methodology similar to that discussed in the previous experiment (Figure 7-2). We evaluated the pain behaviour of two groups of mice following a tibial fracture. For pain management, after the bone fracture, one group received NSAID at the beginning of the active phase, and the other group received NSAID at the end of the active phase.

Immediately after inducing a tibial fracture, all mice increased limb guarding and decreased weight bearing on the injured limb, which are both indicators of pain. Two weeks after surgery, the mice in the group that received NSAID during the active phase recovered the weight bearing in comparison to pre-surgery values (P=0.85, Figure 7-4c). However, mice treated during the resting phase still showed slower recovery after two weeks in comparison to pre-surgery scores in guarding and weight bearing tests ($p<0.05$, Figure 7-4b,c). Interestingly, compared to mice receiving NSAID at the resting phase, those treated with NSAID during the active phase recovered their limb posture and weight bearing faster by more than 40% at day 3 ($p<0.05$, Figure 7-4b,c). Also, the mice treated during the active phase bore significantly more weight on the injured limb at day 14 compared to the resting phase group ($p <0.05$, Figure 7-4c).

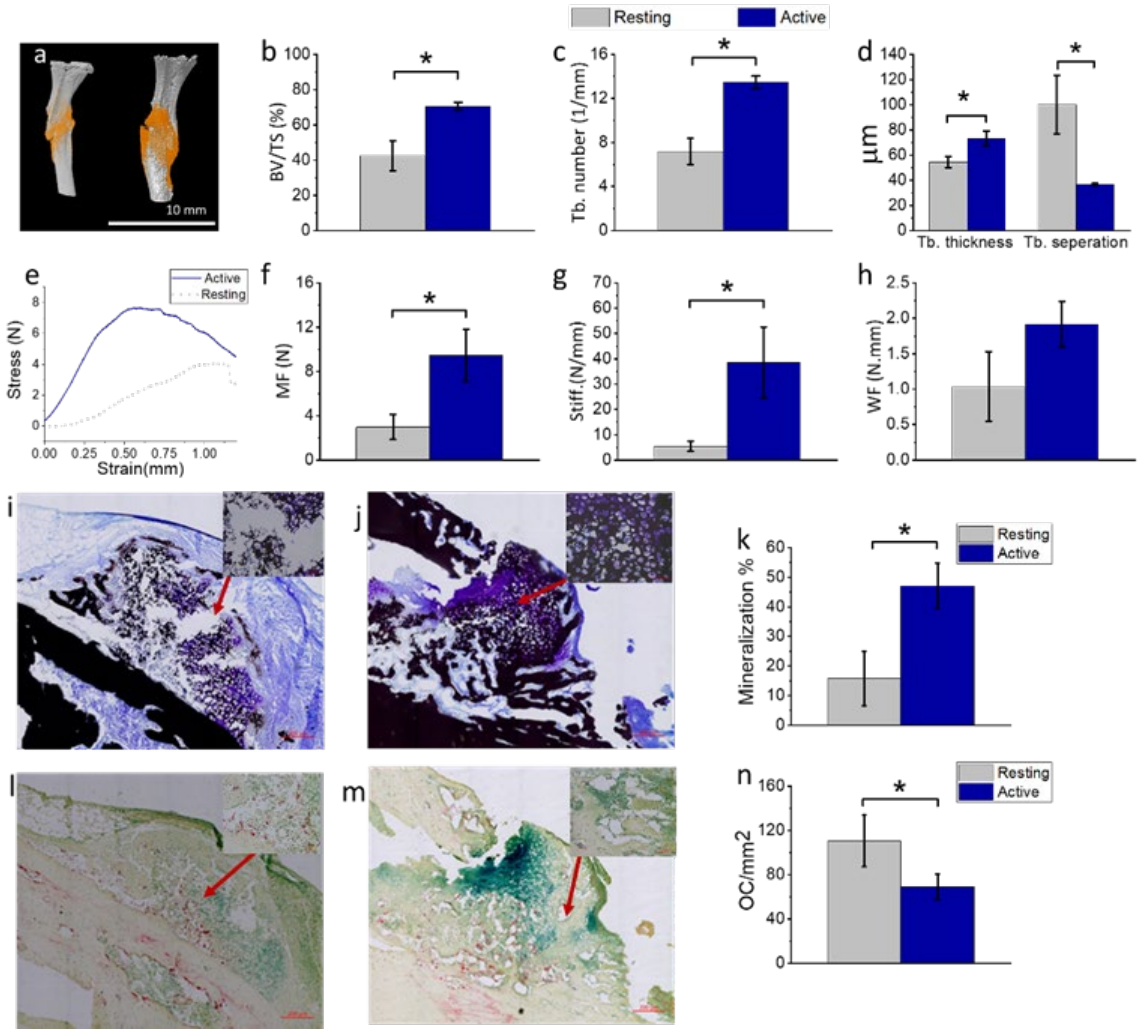
Figure 7-4: Effect of NSAID dose timing (n=8 per group) on pain behaviour assessment at 1, 3, 7 and 14 days after surgery.
(a) Guarding test mesh for the paw of the fractured leg, (b) guarding test, (c) weight bearing test. Scores are expressed as mean ± SE values *P<0.05.



To assess the effect of NSAID timing on bone healing, we analyzed the morphology and mechanical properties of the harvested bones from the mice of the two groups described above. Two weeks after inducing the bone fracture, μ CT analyses revealed that NSAID treatment during

the active phase resulted in significantly larger callus at the fracture sites, and higher bone volume to tissue volume, trabecular number, and decreased spacing between the trabeculae, than NSAID treatment during the resting phase (Figure 7-5b,c,d)($p<0.05$). Histomorphometric analysis of the fractured bones also revealed a significantly larger callus area and greater mineralized bone area in mice treated during the active phase compared to the resting phase (Figure 7-5k), This was associated with significantly fewer tartrate-resistant acid phosphatase (TRAP)-stained osteoclasts in the animals receiving NSAID during the active phase (Figure 7-5n). To investigate whether differences in biomechanical properties accompanied the difference in the structure of the healing callus, the biomechanical characteristics of the healing bones from the two experimental groups were investigated by three-point-bending tests (Figure 7-5e,f,g,h). Analysis of the load-displacement curves showed higher maximum force to fracture and stiffness in the fractured bones of the animals treated during the active phase, in comparison to the mice receiving treatment during the resting phase (Figure 7-5f,g,h).

Figure 7-5: Effect of NSAID dose timing on bone healing outcomes.
(a) µCT images of tibiae retrieved from the NSAID resting time group (left) and NSAID active time group (right) at day 14 after tibia fracture surgery. The group that received NSAID at the active time had (b) a higher bone volume fraction (BV/TV) and (c) trabecular number (Tb.N), (d) lower spacing between the trabecula (Tb.Sp) and higher trabecular thickness (Tb.Th) compared to the group receiving NSAID at resting time (n=7 per group). (e) The mechanical stress-strain curve of 3-point bending tests on the fractured bones, (f) maximum force (MF), (g) stiffness (Stiff.) and (h) work to failure (WF) of the fractured tibiae of each group (n=7 per group). (i-n) Histology Von-Kossa-stained sections: (i,j) mineralization in black at fracture sites in mice (i) receiving NSAID during resting time or (j) during active time. (k) Percentage of mineralized tissue within the callus. Tartrate-resistant acid phosphatase (TRAP) stain (l,m) shows the osteoclasts in the fracture site of mice receiving NSAID (l) during resting time and (m) active time (scale bars represents 200 µm (big image, and 10 µm small image)). (n) Number of osteoclasts per unit area of callus (n=4 per group). All data are expressed as Mean ± SE values. *P< 0.05.



The timing of NSAID administration affects systemic inflammation after bone fracture

To understand how the timing of NSAID administration after bone fracture affects the systemic inflammatory response, we measured the levels of 20 inflammatory cytokines in serum. These analyses revealed increased levels of interleukin IL-13, vascular endothelial growth factor, and IL-4, and decreased levels of IL-1β and interferon gamma-induced protein 10 in the mice receiving treatment during the active phase compared to those receiving it during the resting phase ($p<0.05$) (Figure 7-6a,b).

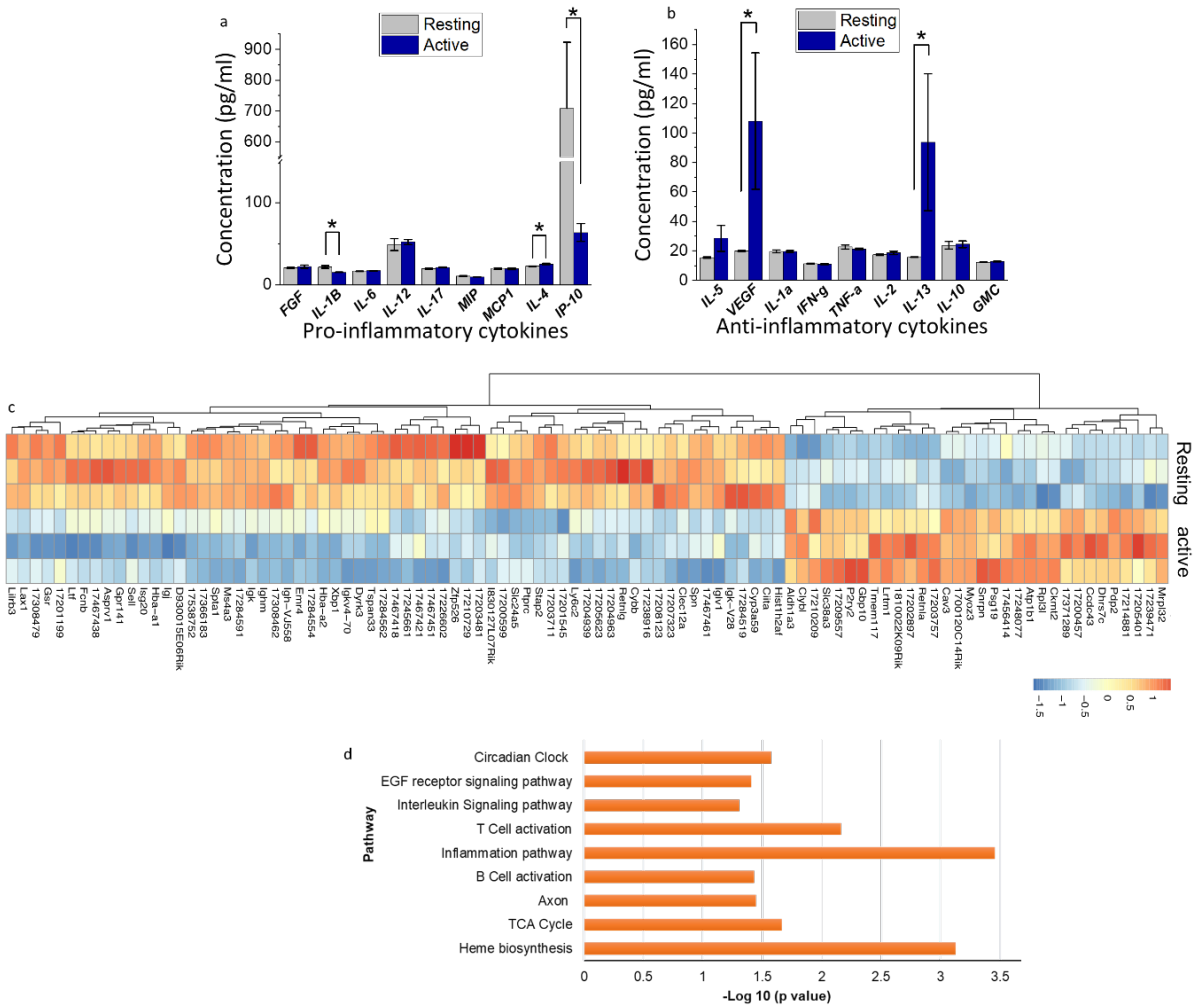
The timing of NSAID administration affects gene expression in bone fractures

The effect of the timing of NSAID administration on the expression of genes at the bone fracture site was assessed using RNA microarray. Extracted total RNA pools were harvested on day 3 after surgery and examined for global gene expression profiles (Figure 7-6c,d and appendix II-Figure 13-3Sa,b,c and appendix II- Table 13-3S and Table 13-4S). NSAID administration during the active phase elicited a different gene expression profile in comparison to the resting phase group. In comparison to the negative control group (no NSAID and no surgery), there were 8300 genes expressed in the group that received NSAID during the active phase after bone fracture, and 7200 genes expressed in the group that received NSAID during resting time after bone fracture (Figure 13-3Sa,b)(Data Set-Series GSE126648-GEO Repository). In total, 555 genes exhibited a significant difference in expression of ≥ 1.5-folds ($p<0.05$) between the two groups (Figure 7-6c), including specific genes involved in the bone healing process and macrophage polarization, such as CCl12, Rental, CCl3 and FGF growth. In addition, clock genes such as Per2 were upregulated by NSAID administration during the active phase. Moreover, genes known to impair bone healing, such as STAT1, were downregulated in the active phase group (Appendix II- Table 13-4S).

According to a ‘gene ontology’ classification, the genes affected by the timing of NSAID administration were mainly related to cellular, metabolic and biological regulation processes (Appendix II- Table 13-3Sand Figure 13-1Sa, b). Also, they were associated with nine different signalling pathways, such as the circadian clock, inflammation mediated chemokines and cytokines, epidermal growth factor receptor (EGFR) pathway, interleukin, T cell activation, B cell activation, axon, TCA cycle, and heme biosynthesis signalling pathways (Figure 7-6d). Among these pathways, the circadian clock and EGFR signalling pathways were expressed in the group that received NSAID during the active phase and not in the resting group.

Figure 7-6: Effect of NSAID dose timing on systemic inflammatory cytokines and gene expression of the healing callus.

(a,b) The concentration of serum cytokines during fracture healing: (a) serum concentrations of pro-inflammatory cytokines, and (b) anti-inflammatory cytokines and growth factors. (c) Gene expression heat map for the top 100 genes that showed at least 1.5-fold difference (P<0.05), upregulated (red) or downregulated (blue), at day three after fracture surgery for the group receiving NSAID at active time compared to those receiving NSAID at resting time. (d) PANTHER pathway ontology analysis showing significantly enriched Panther pathways (p < 0.05) of differently expressed genes between the group that received NSAID at the active time compared to resting time at day 3 after fracture surgery. Concentrations are expressed as mean ± SE values. *P < 0.05.



Discussion

Our results suggest that NSAID administration during the daily activity period results in better postoperative healing and recovery in a bone surgery model. This is likely due to the impact of NSAID timing on inflammation at the healing site, especially through the overexpression of circadian clock genes such as Per2. This effect of NSAID chronotherapy seems to be independent of the time of surgery.

The timing of NSAID administration affects the expression of clock genes

The expression of the genes involved in the circadian clock pathway and circadian rhythm biological process was affected by NSAID chronotherapy (Figure 7-6d). Clock genes such as Per2, Nr1d1, and Nr1d2 appeared to be upregulated in the fracture sites of the group that received NSAID during the active phase, in comparison to the resting phase group. This effect of NSAID chronotherapy is probably through the COX-independent pathway. NSAID administration has been shown to affect Per2 clock gene expression in canine cancer cell lines (*36*). Other studies have shown that independent of COX pathways, NSAID administration affects a variety of transcription factors that regulate Per2 clock gene expression, such as EGR-1 (37, 38), B-Catenin/TCF (39), NF-κB (40), ATF3 and ATF4 (41). Some of these transcription factors were expressed in both experimental groups in comparison to the control group.

Our findings show that NSAID administration at a specific time of the day determines its effect on certain clock gene like Per2.

There is an increasing interest in the possibility of targeting of the circadian clock in therapeutic approaches to control inflammatory diseases, metabolic diseases, and cancer; in addition to promoting healing and maintaining homeostasis (42, 43). Our gene ontology analysis of the differentially expressed genes suggested that the timing of NSAID administration after bone fracture affected several signalling pathways such as: inflammation mediated pathway, interleukin, T-cell and B-cell activation signalling pathways, and EGFR signalling pathway. Studies have demonstrated the effect of these pathways (44-46) on regulating cytokine signaling and the immune response.

RNA microarrays of the fracture site among the group that received NSAID during the active time revealed an upregulation of genes signalling cytokines associated with the polarization of macrophage cells from M1 phenotype to M2 such as Rentla, FIZZ1 and IL-4R1 during bone healing (47). This group also showed expression of genes associated with the recruitment of mesenchymal stem cells and the initiation of the angiogenesis process such as CXCR4 and CCL12 (44) (Appendix II- Table 13-4S). In addition, the group that received NSAID during the active phase demonstrated downregulation of transcripts associated with the expression of pro-inflammatory cytokines including Stat1, IL-9, IL-6ra, and IL-18 (48, 49).

The timing of NSAID administration affects the systemic inflammatory response

Bone healing involves an early inflammatory phase in which immune cells such as neutrophils, natural killer cells (NK) and macrophages, as well as a variety of cytokines, initiate the healing cascade. Cytokine expression varies throughout the post-fracture bone healing period; early stages of bone fracture healing present with inflammatory cytokines that are eventually replaced

by anti-inflammatory cytokines (50). It has been demonstrated that macrophages regulate the recruitment and activation of mesenchymal stem cells by secreting cytokines such as IL-1β, IL-6, and TNF-α during the early period of the inflammatory phase (51). In the subsequent period, the mesenchymal stem cells immunoregulate the macrophages toward anti-inflammatory phenotypes through a COX-dependent pathway that involves the production of PGE2. Afterwards, M2 macrophages produce anti-inflammatory cytokines such as IL-4, IL-10, and IL-13 along with oncostatin M (OSM) to induce osteogenesis, and thus, inflammation is dampened and the tissue repair process is initiated (51-53). The resolution of inflammation is a prerequisite for efficient tissue repair (54). Prolonged inflammatory reactions associated with elevated levels of pro-inflammatory markers appear to delay revascularization and affect the healing outcome (*54-58*).

We found signs of a prolonged inflammatory phase in the group that received NSAID at resting time, while there was an expression of anti-inflammatory cytokines (IL-13, IL-4 and angiogenic factor VEGF) and a decreased level of pro-inflammatory mediators and chemokines (IL-1β and IP-10) at day 3 following surgery in the group that received NSAID during the active phase.

The COX enzyme, the key target of NSAID, plays a vital role in the generation of the inflammatory response during the bone healing process by converting arachidonic acid to prostaglandins (9). Also, the COX enzyme and PGE2 have been found to affect the response of immune cells such as macrophages during bone healing in the synthesis and release of pro-inflammatory and anti-inflammatory mediators (59-61). Our study indicates that administration of NSAID during the active phase could modulate the synthesis and release of these cytokines by immune cells (e.g., macrophages) through the COX-inhibition pathway, decreasing pro-inflammatory cytokines (e.g.,

IL-1β and interferon gamma-induced protein 10) and increasing anti-inflammatory cytokines (e.g., IL-13 and IL-4) (Figure 7-6a,b).

The timing of NSAID administration affects bone fracture healing and recovery

NSAID administration during the active phase after bone fracture improved bone healing by increasing the percentage of mineralization tissue at the fracture site and decreasing the number of osteoclast cells, which resulted in increased bone strength and better postoperative recovery. This effect on bone metabolism was probably a result of COX-dependent and COX-independent inflammatory pathways affected by NSAID chronotherapy.

Results from recent studies showed that endochondral ossification in fracture healing involves the circadian clock genes, especially Per2 (21). Taken together, the differential expression of the Per2 clock gene and the over-representation of the circadian clock pathway along with other inflammation and immune pathways suggested an immune and inflammatory response in the mice that received NSAID during the active phase that may improve bone healing. Also, the microarray gene expression and cytokines analysis suggested that the timing of NSAID administration after bone fracture stimulated the healing process in the group that received NSAID during the active phase. Indeed, we observed an earlier resolution of the inflammatory process, which was manifested by an anti-inflammatory state (increased serum levels of IL-13, IL-4 and VEGF) mainly due to the expression of genes that are involved in the polarization of macrophage cells toward anti-inflammatory phenotype M2, which is associated with better healing outcomes (*53*). Schmidt and Serhan demonstrated that between the first day and day three after trauma, the upregulated anti-inflammatory signalling coincides with the

overexpression of angiogenic factors such as VEGF in the hematoma of the bony environment (54, 56).

The M1 macrophage is associated with bone destruction and M2 cells with tissue repair. M2 macrophage cells produce anti-inflammatory cytokines such as IL-10, IL-13, and IL-4 and promote osteogenic differentiation of bone marrow-derived mesenchymal stem cells, the precursor of the osteoblast (62). The differentiation of mesenchymal stem cell to osteoblast requires direct cell-cell contact with macrophage cells (M2 phenotype), and the production of a soluble factor known as oncostatin M. Oncostatin M production from this cell-cell contact is regulated by prostaglandin E2 and cyclooxygenase 2 loops (63).

Materials and methods

Study design

The primary objective was to estimate the effect of timing NSAID administration on pain and bone fracture healing. In the first experiment, we assess the effect of timing of bone fracture surgery in mice; the second experiment was to test the effect of NSAID administration during the active period compared to resting phase after bone fracture surgery in mice on pain and postoperative recovery. The third experiment was to identify serum cytokines and different gene expression from the healing callus of a fractured bone in mice between those animals who received NSAID at a different time of the day. For all experiments, replicate numbers are outlined in Materials and Methods or figure legends. Minimum sample size was determined by power analysis based on an estimated. Mice in all experiments were age-matched and randomized into

groups. Experimenters were blinded to experimental groups to remove bias. Adverse animal welfare issues were sufficient to halt experiments but did not arise during this work.

The effect of the timing of bone surgery on bone healing and recovery

To assess the effect of the timing of bone fracture on postoperative pain and bone healing, we used a tibia-fracture model in mice. In this model, animals underwent bone fracture surgery at two different times of the day (active and resting time). Behavioural indices of pain, including weight-bearing and guarding tests, as well as the biomechanical and histomorphometric properties of the healing bones, were examined.

Animal model

After obtaining the ethical approval obtained from the Animal Care Committee at McGill University, we acquired sixteen 4-months-old wild-type C57BL/6j mice weighing 25 to 28g from Jackson Laboratories (Bar Harbor, ME). We chose this strain, which is deficient in melatonin, to identify the pathways independent of the melatonin systemic effect (64). The mice were housed in pathogen-free conditions at 22°C with a 12-hour alternating light/dark cycle fed on water ad libitum.

Experimental groups

Cage assignment, mouse numbering and allocation to study groups were performed by a researcher not otherwise involved in the study procedures. Mice were randomly assigned to one of two study groups following an automated process using Experimental Design Assistant from the National Centre for the Replacement and Reduction of Animals in Research.

One group received open tibia-fracture surgery at the beginning of the resting phase corresponding to zeitgeber time ZT2, where ZT0 refers to the time the lights turn on in the animal

facility (6:00 am). The second group had the same surgery at ZT13, which corresponds to the beginning of the active phase when the light is off (from 6:00 pm onwards). Mice are nocturnal animals in which the active phase starts at the beginning of the night and ends in the morning. Before the surgical intervention, mice were acclimated for two weeks to the environment and the facility and exposed to behaviour testing equipment twice before obtaining baseline measures.

Surgical intervention

For our study, we used the open osteotomy fracture model developed by Grestenfield et al. (*65*). All surgeries were performed by the same operator who was not involved in the randomization, allocation concealment process or outcome measurements. Anesthesia was induced initially with a 4% isoflurane/oxygen mixture and maintained at 2%, and a buprenorphine (5 %) subcutaneous injection was given before surgery for pain control. An incision centred over the knee joint medial to the patellar ligament was done to create an entry portal to the tibia medullary canal using a 27-gauge x ½ inch TB syringe. The stylet of a 25-gauge spinal needle was inserted in an intergrade fashion through the lumen of the 27-gauge needle down to the distal growth plate, and its tip was used to bend the spinal needle wire, which was cut 1 mm above the bent, then was inserted and adapted smoothly beneath the patellar pliers. The wound was closed with a 5-0 absorbable vicryl suture. Mice were monitored for any signs of improper healing or infection. All animals were euthanized two weeks later at the same time of the day.

Pain assessment

Behavioural indices of pain including weight bearing and guarding, were assessed at baseline (pre-surgery), and on day 3, 7 and 14 after fracture surgery. All assessments were performed at

the same time of the day between 11:00 am and 1:00 pm by an investigator blinded to the experimental design and intervention group. Detailed materials and methods are provided in the supplementary information of materials and methods- appendix II.

Assessments of the healing bones

Following euthanasia, the harvested tibiae were examined by microcomputed tomography (μ-CT), biomechanical testing and histomorphometric analyses. Detailed materials and methods are provided in the supplementary information of materials and methods- appendix II.

The effect of the timing of NSAID administration on pain and bone healing

To assess the effect of the timing of NSAID administration on bone healing and pain, we conducted an experiment using 16 mice. The housing and handling of mice and the surgical intervention were performed as described above.

Two study groups of mice were randomly assigned; one group received fracture surgery and postoperative subcutaneous injections of NSAID (carprofen 20mg/kg) only at resting time (ZT2) for three days, whereas the second group had the same treatment at the active time (ZT13). The carprofen dose is equivalent to a typical postoperative prescription of ibuprofen of 500 mg every 8 hours (*66*). Mice were observed for any signs of improper healing or infection. All animals were euthanized two weeks after surgery.

Pain behaviour assessments after bone fracture surgery and the examination of the harvested bones were carried out as described above. Details are provided in the supplementary information of materials and methods- appendix II.

Identify inflammatory cytokines and metabolic pathways affected by the timing of NSAID administration after three days of bone fracture surgery

To address this aim, we performed a third experiment with nine mice randomly assigned to three groups. One group received an NSAID treatment for three days after bone fracture surgery during resting time (ZT 2) and the second group received the same treatment during active time (ZT13), while a third group did not receive any surgery or NSAID. Surgery was performed for the NSAID groups as described above at the same time of the day for all mice. Mice were observed for any signs of improper healing or infection after surgery. All animals were euthanized after three days.

Assessment of early healing at the molecular level

We performed a serum cytokine analysis and RNA microarray sequencing of the healing callus three days after surgery. Detailed materials and methods are described in the supplementary information of materials and methods- appendix II.

Statistics

The sample size for the groups of the first two experiments was calculated on o.8 power, based on a coefficient of variation of 25% in the types of data that are collected, and after accepting α and β errors of 5%. All data expressed as mean ± SE. The normal distribution of the data was checked using Shapiro-wilk test. For comparison between two groups, unpaired Student's t-tests used when the data was normally distributed if not Mann-Whitney test was performed. Two-way ANOVA analysis followed by Fisher LSD post hoc test used for comparison between groups at different time points. Statistical analyses were performed using OriginPro 2017 (OriginLab, Northampton, MA). Differences were considered significant at $p < 0.05$.

For the cytokines experiment, a sample size of 3 with three replicated was used to achieve an $\alpha=0.05$ and a power of 0.8 according to previous studies (67, 68). Data not normally distributed were analyzed using Mann-Whitney test. OriginPro 2017 (OriginLab, Northampton, MA). For

gene expression data, the PANTHER classification systems (PANTHER overrepresentation test, Reactome Pathways, version 11(2016), www.pantherdb.org) with the whole mouse genome (data updated to NCBI's January 10, 2011 release) (69). Differences were considered significant at $p < 0.05$.

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Acknowledgments: We thank the Bone Biology Lab, McGill University for providing the space and the scan to perform the CT scan images. Also, we thank the McGill Quebec Genome Center for their help with the gene expression analysis experiment. Dr Tamimi and Nicolau hold a Canada

Research Chair. **Author contributions:** H.A., L.A., Q.G., M.N.A, A. M., A. A. performed the experiments. H.A, L.S., B.N., and F.T. designed the experiments and wrote this manuscript. **Competing interests:** The authors declare that they have no competing interests.

A preface to manuscript III

The following chapter addresses the thirds objective of this thesis, which is to design and launch a pilot RCT to test a new treatment protocol of using NSAID after surgical third molar extraction based on circadian rhythm concept in human. This research will help to provide primary data about the effect of timing NSAID administration on healing outcome after small oral surgical approach that includes bone removal. Moreover, this study can help in the preparation of the main trial that may include other surgical procedures. Bibliography of this manuscript is incorporated at the end of the chapter.

8 Manuscript III

Efficacy of Non-Steroidal Anti-Inflammatory Drug (Ibuprofen) Chronotherapy in Healing After Surgical Extraction of the Mandibular Third Molar – A Randomized Controlled Trial Protocol

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Abstract

Background: Clinical and preclinical studies have demonstrated encouraging results of non-steroidal anti-inflammatory drugs (NSAID) chronotherapy in the management and treatment of inflammatory diseases such as rheumatoid arthritis. However, no previous clinical trials have addressed how the timing of NSAID administration within the day affects pain and healing outcomes after oral surgery that involves bone removal, such as surgical extraction of the third molars.

Methods: To address our aim, we designed a single-center double-blind, randomized controlled trial. Patients who need a lower third molar extraction and meet the eligibility criteria will be recruited. Participants will be randomized into two groups. Subjects in group one will be instructed to take NSAID (ibuprofen 400 mg) at 9 AM and 2 PM combined with a placebo before bed between 9 PM for three days postoperatively. Subjects in group 2 will be instructed to take NSAID (ibuprofen 400 mg) between 9 AM, 2 PM and 9 PM for three days postoperatively. The patients' self-reported pain in the three days after surgery will be recorded as the primary outcome. Additionally, healing indicators such as the maximum interincisal distance and measurements of facial swelling will be recorded preoperatively and four days postoperatively. Each participant's blood level of C-reactive protein will be recorded pre- and postoperatively as an inflammatory marker.

Discussion: The study will estimate the effect of using NSAID only in the morning and early afternoon following surgical extraction of the third molar to decrease pain and improve postoperative healing and recovery in comparison to the routine use of NSAIDs three times per day.

Trial registration

The trial was registered with ClinicalTrials.gov in November 2018 (No. NCT03789058).

Introduction and background

The surgical extraction of wisdom teeth under local anesthesia is one of the most common oral surgical procedures (1). Each year more than 5 million patients in the USA having third molars (wisdom teeth) at an annual cost of over $3 billion. Additionally, more than 11 million patient-days of "standard discomfort or disability" were reported due to surgical removal of wisdom teeth, with an average of 4.9 lost work days per procedure (2, 3).

Most of the wisdom teeth surgical extractions require bone removal (4). Removing bone during extraction is associated with pain and discomfort related to the inflammatory process after surgery.

Currently, postoperative pain management is limited to acetaminophen, opioids and NSAIDs (5, 6). However, these drugs are all problematics. Acetaminophen is not effective in managing severe pain (6). Opioids and NSAID are effective in pain management, but opioids can cause constipation and addiction (7, 8), While NSAID may delay bone healing (9, 10).

Dentists and maxillofacial surgeons all over the world prefer to prescribe NSAID after this type of surgery (11). The mechanism of action of NSAID is the reversible inhibition of the enzyme cyclooxygenase (COX), which is believed to be responsible for the synthesis of prostaglandins (10). Prostaglandins play a major role in inflammatory and nociceptive processes (12). Two isoforms of COX, namely, COX-1 and COX-2, are responsible for the synthesis of Prostaglandin from arachidonic acid. Both have essential roles in the inflammatory process after bone surgery,

but COX-1 is involved more in the integrity of the gastrointestinal tract and renal tract tissue, while COX-2 is mainly involved in the inflammatory and healing process later (13).

NSAIDs are either nonselective (inhibiting both COX-1 and COX-2) or selective (inhibiting COX-2 only). Ibuprofen is a peripherally acting analgesic works on inhibition of both COX-1/COX-2 inhibition, it provides fast analgesic effect without any increasing side effects risk (14). Ibuprofen is routinely used in the treatment of moderate to severe acute pain such as dental pain or postoperative discomfort (15-17).

Results from Cochrane systematic reviews demonstrate that higher doses and frequencies of NSAID use are associated with better pain control after dental (wisdom tooth extraction) and non-dental surgeries (9, 18, 19). Increasing the dose and frequency of NSAIDs was associated with an increased risk of adverse effects. Despite the promising results associated with the use of different NSAIDs for pain control after wisdom tooth surgeries, patients still report pain and other discomfort indicators such as swelling and trismus, especially during the first three days after the procedure (20). These symptoms can affect the patients' activities of daily living and even quality of life (3, 20-22). Animal (23-35), retrospective, and some clinical studies (36-40) suggest that NSAID affect bone healing outcomes.

All living organisms possess a circadian rhythm that anticipates the response to changes during the 24-hour cycle (41). The circadian system in mammals is composed of a central clock within the suprachiasmatic nuclei and a peripheral clock inside all cells (42). This molecular clock modulates the immune response and bone healing process (42). Macrophages activity, leukocytes recruitment, and proinflammatory mediators such as interleukin-1β (IL-1β), IL-6, and IL-12 increased at the beginning of the daily active phase. During the active phase, there are also

increased levels of Toll-like receptors TLR9 and TLR4, which upregulate the expression of CCL2, CXCL1, and CCL5, leading to leukocyte recruitment and potential tissue damage at the injured site (41-44). On the other hand, anti-inflammatory mediators and other growth and angiogenesis factors, such as vascular endothelial growth factor (VEGF), peak during the resting phase (42, 45, 46).

The 24-hour circadian rhythm oscillation occurs in bone tissue during growth (47), formation, resorption (48, 49), and endochondral ossification during fracture bone healing (49). Experimental studies in rodents and humans reveal that disruption of sleep and circadian rhythm impair bone formation (50). All bone cells, such as osteoblasts, osteoclasts, and chondrocytes express clock genes that influence bone volume regulation, such as Per or Cry (51, 52).

The circadian clock also affects pain, with sensitivity peaking during the active phase (53). Part of the pain response oscillation could be explained by changes in COX-1 and COX-2 activity throughout the day (54), especially after an injury or insult (55). These variations may contribute to the clinical evidence of circadian and circannual variations in the pharmacokinetics effects of NSAID. Specifically, maximum absorption and effectiveness are achieved when the drug is administered during the active phase (56-60).

The literature outlined above has led to the development of chronotherapy, which is the science of preventing or treating illness according to biological rhythms (61). It involves the timing of pharmacological, medical or surgical interventions to minimize side effects and increase treatment efficacy (56-58). More than half of the available medications, including some of the overcounter ones, their action is regulated by circadian clocks which make the timing of administration a promising treatment strategy (62). Although this essential biological process has

been largely ignored, many drugs may achieve maximum absorption and effectiveness when administered during the active phase (53). The optimal time of the day for administration depends on the species, i.e., daytime for humans but night-time for several nocturnal animals (e.g., mice).

Recent evidence suggests that the timing of skin wounds or surgery affects healing and postoperative recovery after cardiovascular surgeries (63, 64).

The chronotherapeutic use of anti-inflammatory medications after oral surgeries is of clinical importance, especially with increasing evidence regarding the clinical efficacy of this approach in medicine. No clinical studies have investigated the effectiveness of this approach after minor oral surgeries.

Within the limitations of the existing literature, we concluded that NSAID delay bone healing in animals. Interestingly, of previous studies tested the effect of NSAID chronotherapy on post-surgical pain and bone healing. Indeed, none of the articles included in our systematic review reported the time of day at which NSAIDs were administered or the time (day or night) that surgery was performed.

This gap, combined with burgeoning evidence in the medical literature suggesting the effectiveness of chronotherapy, motivated us to undertake a series of animal studies investigating the effect of chronotherapeutic NSAIDs administration on bone healing. Results of our preclinical studies showed that administering NSAIDs at wake-up compared to bedtime cut in half the recovery time for pain and bone healing in mice, including mechanical and histomorphometric properties of the healing callus. These findings support the role of the

circadian rhythm in inflammation during bone healing and may have major implications for bone-related surgical interventions.

Although chronotherapy has the potential to better control inflammation, decrease pain and recovery time, no clinical studies have yet investigated the effectiveness of the timing of NSAIDs administration on healing outcomes after surgery involving bone removal in humans. Therefore, we propose to address this gap by conducting an RCT to investigate the chronotherapeutic use of NSAIDs in humans following a validated model of minor surgery involving the bone for postoperative pain and pain management.

Expected contribution and research question

The overarching aim of this study is to contribute evidence regarding the efficacy of NSAID chronotherapy for postoperative pain and pain management after all bone related injuries or surgeries by using validated surgical model such as a third molar surgical extraction. This is an important aim considering that the outcomes of this investigation can be used for other medical interventions involving bone healing. The research question is as follows: Among patients undergoing 3rd molar extraction surgery, does a novel protocol for post-surgical pain based on chronobiological principles decrease pain levels and improve bone healing compared to the current standard of care?

Objectives

Working hypothesis: Among patients undergoing surgical third molar extraction, the administration of NSAID during morning and early afternoon only will result in better postoperative recovery than the routine standard care administration of NSAIDs for three times per day. The specific objectives of this study are (i) to assess the effect of ibuprofen 400 mg on

postoperative pain severity and wound healing indicators such as edema and mouth opening and (ii) to compare the levels of inflammatory indicators such as C-reactive protein (CRP) three days after surgical third molar extraction between those receiving ibuprofen 400 mg in the morning and early afternoon to those receiving it three times a day.

Methods

A flow chart of this study is shown in (Appendix III-Figure 14-1S). The schedule of enrollment and intervention is presented in (Appendix III-Table 14-1S).

Study location

The current project is a single-center double-blind randomized controlled trial (RCT). Randomization will take place at the individual level. The study will be carried out in the Dental Teaching Clinics at the Jordan University of Science and Technology in Irbid, Jordan.

Ethical approval

The study obtained approval from the Institutional Review Board of Jordan University of Science and Technology in Irbid, Jordan (protocol number 393/2017) and is registered in the ClinicalTrials.gov clinical trials registry (No. NCT03789058) to be conducted in the University's Dental Teaching Clinics in Irbid, Jordan.

Study participants

Subjects referred for third molar extraction at the Oral and Maxillofacial Surgery Department, Jordan University of Science and Technology (JUST), Irbid, Jordan, will be approached for recruitment. The maxillofacial specialist will examine all patients clinically and evaluate their radiographs to determine their suitability for inclusion in the study.

Subjects who have been indicated for third molar extraction and fulfill the study criteria will be informed about the research, including its nature and purpose. Those interested will be invited

to take part in the study. Each patient will be asked to sign a surgical procedure consent form on the day of surgery.

Inclusion and Exclusion

To be considered eligible for the study, the individual must (i) be aged between 18 and 35 years old, (ii) be healthy according to the American Society of Anesthesiologists (ASA) classification (subject should not have an active infection, trismus, hyperthermia, or swelling before surgery and must be able to maintain adequate oral hygiene), (iii) have an adequate understanding of written and spoken English or Arabic to fill out a questionnaire, and (iv) be capable of signing an informed consent form. In addition, in the interest of standardizing the clinical cases of third molar surgical extraction, the indicated teeth should be lower third molars which are bony impacted. People will not be eligible for the study if they (i) have a history of systemic diseases (e.g., diabetes mellitus, hypertension, gastric ulcer), (ii) have a severe/serious illness that requires frequent hospitalization, (iii) are current smokers, (iv) are pregnant or breastfeeding, (v) are taking anti-inflammatory or analgesic drugs in the previous two weeks or are allergic to NSAIDs, (vi) have impaired cognitive or motor function, or (vii) are unable to return for evaluations/study recalls. Patients who develop a postoperative infection will be dropped from the study.

Clinical procedure and experimental interventions

For each surgery, a full thickness flap will be lifted after giving local anaesthesia (2% lidocaine with 1:100,000 epinephrine). A no. 701 surgical bur will be used to perform osteotomy. The surgical site will be inspected after extraction, and any sharp bone will be filed to prevent discomfort. Copious irrigation will be applied, followed by closure using 3/0 polyglactin910 (Vicryl) sutures. Tooth location (right or left), the volume of local anesthesia administered (the

number of anesthetic tubes used), the time of the surgery (morning only), and the time needed to perform the surgical procedure will be recorded. Each participant will receive an appropriate dose of ibuprofen 400 mg for three days postoperatively. Group 1 will receive two doses of ibuprofen before afternoon, as well as a third dose consisting of a placebo (a sugar tablet of the same colour and shape as the active drug tablet) at bedtime; group 2 will receive three doses of ibuprofen 400 mg three times per day. The oral medications (ibuprofen and the placebo) are identical in appearance and will be given to each patient by the investigator in a closed envelope labelled with the patient's assigned study identification number, known only by the investigator.

Blinding and randomization

In the interest of minimizing bias, both the patient and the surgeon will be blinded to treatment group allocation. After undergoing clinical evaluation by the surgeon, patients who meet the eligibility criteria will be randomized into two experimental groups. One clinician will perform the surgical procedure for all the participants. Randomization of participants will be completed by an investigator who is not involved in the recruitment or treatment of the participants; the investigator will apply permuted-block randomization via the website www.randomization.com. The investigator will distribute the ibuprofen and the placebo in the appropriate sequence. Other than providing the ibuprofen and the placebo, the investigator will have no contact with the study's participants and no involvement in data collection or analysis. All the records and the results of the tests will be identified with codes known only by a person who does not participate in the clinical trial.

Study outcome measures

The target outcomes are pain relief and healing indicators.

Primary outcome measure

For our primary outcome, pain severity in the first three days after surgery, the patient will record the pain score three times per day using the Verbal Rating Scale (VRS), which has been validated in many clinical trials (65). This scale quantifies the amount of pain that a patient feels and ranges from 0 (no pain) to 10 (the worst pain imaginable).

Patients will be instructed to fill out a journal diary for three days after surgery and deliver the diary to the investigator during the follow-up visit. For ethical reasons, all participants will receive rescue medication (500 mg acetaminophen), and they will be instructed to take two tablets as needed, not to exceed 4 g/day. Patients will be permitted take the painkiller whenever they feel considerable pain, per their judgment, and will be instructed to wait at least 6 hours between doses and write in the diary each time the medication is used.

All participants will be asked to record the types of food they eat for three days after surgery. Additionally, each participant will record any side effects during those days.

Secondary outcome measures

Other target outcomes will consist of healing indicators; in this study, we will use proxy bone-healing indicators including facial swelling and mouth opening. A single investigator will collect these measures at baseline (before the surgical procedure) and on day 4. To assess postoperative swelling, we will measure: a) the distance in millimeters from the bottom edge of the earlobe to the midpoint of the symphysis Hirota, called horizontal distance to the symphysis (DHS); b) the distance in millimeters from the bottom edge of the earlobe to the external angle of the mouth, called: horizontal distance to the corner (DHC), and c) The distance in millimeters from the palpebral outboard angle to the gonial angle, called: vertical distance (DV).

The swelling ratio will be calculated as follows: (postoperative measurement − preoperative measurement) × 100/preoperative measurement. Trismus, an inability to open the mouth, may be a consequence of the surgery due to inflammatory processes involving the muscles. We will evaluate this condition by measuring the distance between the incisal edges of the upper and lower central incisors with a ruler when the mouth is fully open. Trismus will be considered present if the patient has limited jaw opening less than or equal to a 10-mm interincisal distance (65). The presence of erythema, muscle tenderness, tempers mandibular symptoms and dry socket will be checked and recorded during the follow-up visit.

Explanatory outcome measure

The inflammatory condition associated with wound healing will be explored through the expression levels of CRP A venous blood sample of 3 ml will be drawn from the cubital fossa of each patient two times, once in each treatment phase (once preoperatively and once three days postoperatively). The collected blood samples will be analyzed in the JUST laboratory immediately, and the result will be sent to the investigator by email using patient serial numbers. Blood CRP levels will be tested one hour before the surgical procedure.

Statistical analysis

To estimate the sample size for this pilot RCT, we followed the strategy recommended by Whitehead et al.(66) to optimise resources and ensure precision of estimates of variation in the outcome measures. We require 30 participants in each arm to estimate a standardized difference of 0.25 in pain scores between the intervention and control arms with a power of 80% and Type I error rate of 5%. Therefore, a total of 70 participants will be randomized in two arms, taking into consideration a 10% loss to follow-up.

By the end of the study, randomized groups will be revealed, and statistical analysis will be performed as group 1 or group 2. The meaning of the two groups will be known after the analysis. We will focus on obtaining precise estimates of the following parameters of primary outcome measures: i) standard deviation; ii) intra-participant correlation; iii) incidence proportions of binary outcomes; iv) mean differences between randomized groups in the analysis.

Any change to the statistical plan will be accounted for by publication. A detailed statistical analysis plan will be published.

Discussion

The results of this study may reveal part of the role of the circadian rhythm and NSAID chronotherapy in pain and inflammation during wound healing after surgical interventions that include bone removal, such as surgical extraction of the third molars. The findings may change the clinical practice of all bone-related surgical interventions and, at the same time, provide a new, simple way of managing pain and inflammation in surgical patients that will not affect the type of drug used or the cost of treatment.

Our group's animal studies also obtained promising preliminary data, demonstrating that NSAID administration at the beginning of the active phase, compared to the resting phase, is associated with improved recovery from bone fracture surgery.

The results of recent studies (63, 64, 67) on the effect of surgery timing on healing outcomes for skin wounds or heart surgeries, showed how important the internal clock is to the process of healing injuries. The findings from these studies show for the first time how circadian factors are important for wound healing. By taking these into account, it is possible not only to identify novel

drug targets but also to increase the effectiveness of established therapies by changing what time of day they are given to maximize their healing benefits.

In the design of the clinical trial, we have taken into account the pharmacokinetic differences between humans and mice. In mice, carprofen is administered only once a day, but in humans, NSAIDs are often prescribed every 8 hours. Accordingly, in our RCT, the experimental group will receive NSAID treatment only two times per day: once after breakfast and once after lunch, but not at night. The control group will receive conventional NSAID treatment three times per day.

Trial status

This trial has begun receiving and treating patients.

List of abbreviations

AA. arachidonic acid
ASA. American Society of Anesthesiologists
Bmal1. brain and muscle Arnt-like protein-1
CCL2. chemokine (C-C motif) ligand 2
CCL5. chemokine (C-C motif) ligand 5
Clock. circadian locomotor output cycles kaput
COX. cyclooxygenase
CRP. C-reactive protein
Cry. cryptochrome
CXCL1. chemokine (C-X-C motif) ligand 1
IL-12. interleukin-12
IL-1β. interleukin-1β
IL-6. interleukin-6
JUST. Jordan University of Science and Technology
NSAID. nonsteroidal anti-inflammatory drug
Per. period
PG. prostaglandin
TLR4. Toll-like receptor 4
TLR9. Toll-like receptor 9
VAS. Visual Analogue Scale
VEGF. vascular endothelial growth factor

Acknowledgments

We thank Jordan University of Science and Technology in Irbid, Jordan (funding number: 20170307), for supporting this study.

Competing interests

The authors declare no conflicts of interest.

Authors' contributions

HA, FT, ZT and BN provided the idea for the study, established the hypothesis, and wrote and revised the manuscript. ZT and MG made significant contributions to the recruitment of the patients and following them up. The trial is conducted in the Dental Teaching Clinics at Jordan University of Science and Technology. All authors reviewed and approved the final manuscript.

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9 Discussion and conclusion

In this section, I discuss the rationale of the project providing an overview of the previous knowledge about circadian rhythm and NSAID therapy after bone fracture. Then, I describe the results from my project and their contribution to the existing literature. Finally, I address the study's limitations, future directions, conclusions and the clinical implications.

9.1 Rationale

The goal of my Ph.D project is to investigate a new, simpler treatment approach to enhance the analgesic efficacy of NSAID and overcome their potential side effects on healing outcomes based on the circadian rhythm. To address this goal, I used an interdisciplinary approach which included a systematic review and meta-analysis, animal experiments and a protocol for a RCT. From a methodological perspective, it is very challenging to understand the impact of NSAID administration on fracture bone healing in a clinical setup only; several additional factors may affect bone healing (e.g., diabetes, smoking, obesity) making it difficult to establish the effect of NSAID on bone healing in human subjects (92). Animal studies help us to understand the normal bone healing physiological process, providing insight on the effect of NSAID in this process due to the possibility of controlling on the environment (60). However, this type of studies have their own challenges including the choice of specific healing outcome and different animal models, ages, or types and doses of NSAID.

Therefore, in my first paper I conducted a systematic review and meta-analysis of animal studies that investigate the effect of NSAID administration on bone healing. Based on this review, I designed a series of animal experiments aiming to investigate the effect of NSAIDs administration

on bone healing and its outcomes. Using the results of these experiments, I then develop a RCT protocol in human to test the timing of NSAIDs administration after third molar surgery involving bone subjects.

9.2 Summary of the research

9.2.1 Systematic Review and meta-analysis

The results of our systematic review and meta-analysis demonstrated a significantly negative effect of NSAID administration on mechanical bending and bone microstructure properties in comparison to control groups. Moreover, the negative effect of NSAID was not significant in mice, young or female animals. Also, our work identified the need for more controlled experimental studies that overcome the risk of bias and report key information such as randomization measures and sample size calculations. In addition, we observed that all the included studies tested the effect of NSAID on bone healing only in either male or female animals. Moreover, previous systematic reviews on the subject did not formulate a precise research question with specific outcomes, and none included a meta-analysis or risk of bias assessment that would help future hypothesis generation and study design (6, 57, 92, 230).

Therefore, my first project indicated the need to conduct more controlled and high-quality pre-clinical animal studies regarding the effect of NSAID on bone healing. The control measures should include housing randomization, blindness and randomization of outcome measurements considering the timing of NSAID administration. The existence of the circadian rhythm and the effect of circadian clock genes on endochondral ossification, pain and behavioural activity have been proved (128). Therefore, there is a need to add in the guidelines of PRISMA checklist and ARRIVE (Animal Research: Reporting of In Vivo Experiments), the reporting of the timing of drug

administration, outcome measurements and sample collection. This will facilitate the planning of future studies and knowledge translation into the clinical setting.

9.2.2 Pre-clinical studies: animal experiments investigating the effect of NSAID and the circadian rhythm on bone healing

We observed a significant modulation of the inflammation and immune response along with the circadian clock pathways and their genes. Some genes were downregulated while others were upregulated. While these findings are interesting, they need to be validated by using quantitative PCR in future studies. Nevertheless, our study provides the first evidence from microarray gene expression and cytokine analysis of early bone healing events after NSAID administration at a different time of the day. Post-surgical NSAID administration during activity time has a significant effect on the expression of specific genes and pathways. Addressing the circadian clock by NSAID chronotherapy could be an important approach to stimulate healing after bone surgery. Drugging the clock components by certain drugs have been investigated by in vivo studies in the treatment of cancer, inflammatory diseases and metabolic disorders and have been found beneficial (231). One of the rare studies to investigate gene expression in relation to the use of NSAIDs showed that their administration is associated with the upregulation of four genes in anti-cancer therapy. Among the genes expresses is Per2, suggesting that some NSAID effect might be mediated by COX / PG independent pathways (232). Our findings concur with these results; we found that timing of NSAID administration after bone fracture surgery affect Per2 gene expression. There is a need for further studies about the effect of NSAIDs on independent pathways and how it can help the healing process.

Our doses are pharmacologically accepted and, although the number of animals in our study is small, our findings support the testing of NSAID chronotherapy in *in-vivo* models and RCT.

As discussed above, the effect of NSAID administration on bone healing outcomes can vary according to the administration time. It has been demonstrated that certain hormones or medications when they applied in different ways can have a different action on bone healing or hemostasis with anabolic or catabolic activity, mainly through parathyroid hormone and Wnt signalling along with the role of sclerostin and its gene SOST. For example, parathyroid hormone has a dual effect if it is administered continuously it works on the RANKL/OPG ratio action or intermittently it may work on SOST/ sclerostin (22). My Ph.D project added another aspect of dual action for NSAIDs depends on the time of administration, despite that this needs to be investigated further with other pain control medications such as acetominaphine, opioid and other types of NSAIDs, in addition to different surgical models.

Giving support to the chronotherapy hypothesis, studies have also shown that the time of the day of injury or surgery is vital for healing outcomes. For example, it influences side effects, especially after skin wound or burn, and heart surgeries (166, 167, 233).

Our promising results along with those from other groups, demonstrate how circadian factors associated with drug administration or surgery time are essential for wound healing. By taking these factors into account, we could identify novel drug targets and also increase the effectiveness of established therapies by changing the time of day at which existing medications are given.

9.2.3 Protocol development for randomized controlled trial

Findings from our preclinical studies demonstrate that NSAID commonly prescribed for pain after bone fracture surgeries are more useful for both pain reduction and bone healing when used chrono-therapeutically. These findings have a potential clinical significance considering the large number of bone-related surgeries performed in Canada and worldwide annually. As only one example, 51,272 and 61,421 Canadians underwent hip and knee replacement surgeries, respectively, in 2014-15 and these surgeries are greatly on the rise (5-year increases of 20.0% and 20.3%, respectively) (234). However, the current evidence is too limited to allow the design of treatment guidelines based on this promising approach. Therefore, in my Ph.D project I continued on the knowledge translation path by preparing and starting a pilot RCT to investigate a new protocol of NSAID administration after minor surgery involving bone removal in humans based on the circadian rhythm concept.

The extraction of third molars, commonly known as wisdom teeth, is one of the most frequent oral surgical procedures worldwide. Although no Canada-wide data are available for this surgery (235), approximately 5 and 1 million third molar extractions are performed annually in the USA and UK, respectively (225, 236, 237). Because it often requires bone removal and leads to a broad range of postoperative discomforts and complications, NSAID are routinely prescribed after this surgical procedure to manage postoperative pain. These key features make third molar extraction an ideal procedure to test the hypothesis that chronotherapy affects the bone healing process.

Our double-blind pilot RCT is being carried out at JUST. The study, registered in the ClinicalTrials.gov clinical trials registry (No. NCT03789058), has allowed us to assess the feasibility

of study procedures, as well as recruitment and loss to follow-up rates. The pilot trial started in June 2018 and finished in May 2019 when we begin the data analysis. Although the sample size of the trial is small, this is a pilot study and will provide us with a good estimation for sample size of a future RCT testing these hypotheses. This work holds a definite potential for the development of treatment guidelines and therefore, for evidence-based change in the clinical management of post-operative pain and inflammation not only for third molar extraction but for all medical interventions involving bone healing.

9.3 Strengths and Limitations

Several limitations of this work should be considered. For manuscript 1, the meta-analysis of previous experimental studies did not include healing outcomes other than bone fracture (e.g., pain behaviour). The measurements of pain behaviour are subjective and depend on the interpretation of the examiner, which may lead to high heterogeneity among the studies. For this reason, I included studies that measure more objective outcomes of bone healing such as mechanical bending, μ-CT and histological variables. However, the systematic review and meta-analysis benefits from several strengths. First, this is the first review conducted on the administration of NSAIDs on animal bone healing. This rigorous work, conducted in collaboration with the liaison librarian for Life Sciences at McGill University, allowed the synthesis of the extensive literature published on the subject. In addition to identifying key factors influencing the effect of NSAIDs administration on the healing process, we also uncovered a notable knowledge gap: none of the included studies tested the effect of NSAID chronotherapy on post-surgical pain and bone healing, or even reported the time of day at which NSAID were administered or the time (day or night) that surgery was performed. Moreover, this work

revealed the existence of important methodological and/or reporting issues in this body of literature that needs to be addressed in future research.

The knowledge gap identified in manuscript 1 was addressed in the series of animal studies described in manuscript 2. However, these pre-clinical experiments have several limitations. First, we measure bone healing outcomes only at one-time point (14 days after surgery), which represents the time when complete hard callus is formed, and it can give us a good indication for the effect of NSAID on early bone healing. Indeed, Singh et al. in 2011 noted that there is a high inhibition COX-2 enzyme in the fracture site after injury at the baseline; there will be impairment of the healing (238). While we did not measure PGE and COX enzymes before and after fracture or NSAID administration, we randomized the treatment groups and performed the drug administration of all animals at the same time of the day according to their allocated experimental group. Moreover, future studies from our group have been planned to follow the effect of NSAIDs chronotherapy at different time points.

A second limitation regards to testing only one type of NSAIDs', a long-acting NSAID (carprofen), and not ibuprofen which is the most commonly used NSAID. This choice was based on several factors: first, my main objective was to test the timing of NSAID administration at the beginning of the activity period of the animal in comparison to resting on fracture bone healing. Since the carprofen half-life is 10 hours, I was able to administer it either in the morning or the evening, depending on the experimental group to achieve the effective concentration for animal pain control after the surgery. In contrast, Ibuprofen has a short half-life of just 2.5 hours and requires continuous administration in the water or the food of the animal for 2-3 days before the surgery, which is incompatible with the objective of my study.

Despite these limitations, the design of these pre-clinical study was carefully planned including the carefully selected animal model (mice with deficient in melatonin), the number of experiments conducted and their complementarity in terms of variables tested (timing of bone fracture, timing of NSAID administration) and outcomes examined (bone fracture pain and recovery, systemic inflammation, gene expression).

Finally, the RCT protocol presented in manuscript 3 and currently being conducted at JUST is a further step in generating solid evidence to bring the benefits of chronotherapy to patients. As such, its first strength is to build on solid preliminary work that I previously conducted with my colleagues. The proposal includes several methods to protect against sources of bias (e.g., random assignment to study groups, double blinding with RCT logistics run by a third party, identical appearance of NSAIDs and placebo tablets) and investigates several pain and healing outcomes (self-reported pain, healing indicators, blood level of C-reactive protein). Furthermore, this trial is sufficiently powered to provide preliminary data, in addition to critical feasibility information, to design a confirmatory multicentric RCT.

9.4 Public health implications

Bone-related surgeries are common medical procedures for millions around the world at an annual cost that can reach to billions. My PhD project contributes to the public health by providing solid evidence across the research continuum, from synthesizing what is already known in the literature to better design my animal experiments that provide evidence to finally conduct a RCT. If the findings from our preclinical studies are confirmed in the RCT, NSAID chronotherapy could improve the management of post-operative pain and inflammation for bone-related

surgical interventions without affecting the type of drugs currently used or increasing treatment costs.

9.5 Future research directions

To test the NSAIDs chronotherapy effect after surgery in a mouse model with knockout clock genes and see the difference in the healing outcomes to wild animals. It will be important to test our hypothesis in a clinical surgical model that allows for collecting bone healing samples and tissue like early implant placement after extraction and soft tissue healing. Moreover, planning for future collaboration with medical research groups to conduct multicenter RCT.

To maximize the impact of the results; one of our main knowledge translation aims will be to inform professional organizations (e.g., Canadian Dental Association, Ordre des dentistes du Québec, Canadian Orthopaedic Association) and assist them in the development of optimized treatment guidelines concerning NSAID administration following surgery involving the bone.

10 Conclusion

- The Systematic review and meta-analysis data demonstrated that NSAID administration decreases the biomechanical properties of healing bone after fracture surgery in comparison to the control group. Moreover, the larger negative effect was in the fractured tibia, female animals and when the time of assessment was before 21days.
- Results from pre-clinical experiments demonstrated that NSAID chronotherapy can substantially improve postoperative recovery.
- NSAID administration upon active phase after bone surgery in mice promotes post-operative recovery & bone healing in comparison to the administration during the resting phase.
- Several inflammatory mediators & genes especially clock gene (Per 2) had been expressed differently in the group receiving the NSAID upon active phase in comparison to those receiving the same treatment in the resting phase.

11 References

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12 Appendix I – Supplementary materials of manuscript I

Table 12-1S: Protocol of the systematic review and meta-analysis

Protocol registered in SYRCLE website-2017:

https://www.radboudumc.nl/en/research/departments/health-evidence/systematic-review-center-for-laboratory-animal-experimentation/protocols

The link to the published registered protocol of the systematic review and meta-analysis

https://issuu.com/radboudumc/docs/non-steroidal_anti-inflammatory_dru?e=28355229/48255236

Table 12-2S: Search strategy

#	Search Statement
Medline data base (start from January 1st 1946)	
1	exp Anti-Inflammatory Agents, Non-Steroidal/
2	(non-steroid* adj3 (inflammat* or anti-inflammat* or antiinflammat*)).ti,ab,kf.
3	((cyclooxygenase or cyclo-oxygenase or COX-2) adj1 (inhibit* or attenuat*)).ti,ab,kf.
4	(adapalene or ampyrone or antipyrine or apazone or aspirin or bufexamac or carprofen or celecoxib or clonixin or curcumin or diclofenac or diflunisal or dipyrone or epirizole or etanercept or etodolac or fenoprofen or feprazone or flurbiprofen or ibuprofen or indomethacin or ketoprofen or ketorolac or masoprocol or meclofenamic or mefenamic or mesalamine or naproxen or niflumic or olopatadine or oxyphenbutazone or phenylbutazone or piroxicam or salicylate* or sulfasalazine or sulindac or suprofen or tolmetin).ti,ab,kf.
5	(NSAID? or tactupump or galderma or differin or auralgan or paladin or salicylic or floctafenine or meloxicam or nabumetone or napafenac or oxaprozin or tenoxicam or tiaprofenic).ti,ab,kf.
6	or/1-5
7	exp Fractures, Bone/
8	exp "Bone and Bones"/de, in, me, pa, pd, pp [Drug Effects, Injuries, Metabolism, Pathology, Pharmacology, Physiopathology]
9	exp Fracture Healing/
10	exp Bone Remodeling/
11	exp Bone Density/
12	(bone adj3 (broken or fracture* or remodel* or regenerat* or density)).ti,ab,kf.
13	((fracture? or bone?) adj3 heal*).ti,ab,kf.
14	((displace* or open or closed or comminuted or greenstick or transverse or oblique or buckled or pathologic or stress or hairline or compound or traumatic or periprosthetic or linear or spiral or compression or avulsion or impacted or burst or chance or flexion or supracondylar) adj3 fracture*).ti,ab,kf.
15	((holstein-lewis or jefferson or clay-shoveler or holdsworth or hume or essex-lopresti or galeazzi or colles? or smith* or barton* or monteggia or rolando or bennett* or boxer* or duverney or pilon or bumper or segond or gosselin or toddler* or bosworth or maisonneuve or "le fort" or pott* or lisfranc or jones or march) adj3 fracture*).ti,ab,kf.
16	((skull or mandibular or nasal or cervical or rib? or sternal or shoulder? or arm? or humerus or forearm? or ulnar or radius or distal or scaphoid or pelvic or femoral or patella or crus or tibia? or trimalleolar or calcaneal or bimalleolar) adj3 fracture*).ti,ab,kf.
17	or/7-16
18	6 and 17
19	18 not (humans/ not (humans/ and animals/))
COCHRANE LIBRARY/DARE/CENTRAL (started from (All keyword searches are "All Text")	
1	MeSH descriptor: [Anti-Inflammatory Agents, Non-Steroidal] explode all trees
2	(non-steroid* NEAR/3 (inflammat* or anti-inflammat* or antiinflammat*))
3	((cyclooxygenase or cyclo-oxygenase or COX-2) NEAR/1 (inhibit* or attenuat*))
4	(adapalene or ampyrone or antipyrine or apazone or aspirin or bufexamac or carprofen or celecoxib or clonixin or curcumin or diclofenac or diflunisal or dipyrone or epirizole or etanercept or etodolac or fenoprofen or feprazone or flurbiprofen or ibuprofen or indomethacin or ketoprofen or ketorolac or masoprocol or meclofenamic or mefenamic or mesalamine or naproxen or niflumic or olopatadine or oxyphenbutazone or phenylbutazone or piroxicam or salicylate* or sulfasalazine or sulindac or suprofen or tolmetin)
5	(NSAID? or tactupump or galderma or differin or auralgan or paladin or salicylic or floctafenine or meloxicam or nabumetone or napafenac or oxaprozin or tenoxicam or tiaprofenic)
6	#1 OR #2 OR #3 OR #4 OR #5
7	MeSH descriptor: [Fractures, Bone] explode all trees
8	MeSH descriptor: [Bone and Bones] explode all trees
9	MeSH descriptor: [Fracture Healing] explode all trees
10	MeSH descriptor: [Bone Remodeling] explode all trees

11	MeSH descriptor: [Bone Density] explode all trees
12	(bone NEAR/3 (broken or fracture* or remodel* or regenerat* or density))
13	((fracture? or bone?) NEAR/3 heal*)
14	((displace* or open or closed or comminuted or greenstick or transverse or oblique or buckled or pathologic or stress or hairline or compound or traumatic or periprosthetic or linear or spiral or compression or avulsion or impacted or burst or chance or flexion or supracondylar) NEAR/3 fracture*)
15	((holstein-lewis or jefferson or clay-shoveler or holdsworth or hume or essex-lopresti or galeazzi or colles? or smith* or barton* or monteggia or rolando or bennett* or boxer* or duverney or pilon or bumper or segond or gosselin or toddler* or bosworth or maisonneuve or "le fort" or pott* or lisfranc or jones or march) NEAR/3 fracture*)
16	((skull or mandibular or nasal or cervical or rib? or sternal or shoulder? or arm? or humerus or forearm? or ulnar or radius or distal or scaphoid or pelvic or femoral or patella or crus or tibia? or trimalleolar or calcaneal or bimalleolar) NEAR/3 fracture*)
17	#7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13 OR #14 OR #15 OR #16
18	#6 AND #17
EMBASE (OVID)	
1	exp nonsteroid antiinflammatory agent/
2	exp prostaglandin synthase inhibitor/
3	(non-steroid* adj3 (inflammat* or anti-inflammat* or antiinflammat*)).ti,ab,kw.
4	(adapalene or ampyrone or antipyrine or apazone or aspirin or bufexamac or carprofen or celecoxib or clonixin or curcumin or diclofenac or diflunisal or dipyrone or epirizole or etanercept or etodolac or fenoprofen or feprazone or flurbiprofen or ibuprofen or indomethacin or ketoprofen or ketorolac or masoprocol or meclofenamic or mefenamic or mesalamine or naproxen or niflumic or olopatadine or oxyphenbutazone or phenylbutazone or piroxicam or salicylate* or sulfasalazine or sulindac or suprofen or tolmetin).ti,ab,kw.
5	(NSAID? or tactupump or galderma or differin or auralgan or paladin or salicylic or floctafenine or meloxicam or nabumetone or napafenac or oxaprozin or tenoxicam or tiaprofenic).ti,ab,kw.
6	((cyclooxygenase or cyclo-oxygenase or COX-2) adj1 (inhibit* or attenuat*)).ti,ab,kw.
7	or/1-6
8	exp *fracture/
9	exp *bone/
10	exp bone remodeling/
11	exp bone density/
12	(bone adj3 (broken or fracture* or remodel* or regenerat* or density)).ti,ab,kw.
13	((fracture? or bone?) adj3 heal*).ti,ab,kw.
14	((displace* or open or closed or comminuted or greenstick or transverse or oblique or buckled or pathologic or stress or hairline or compound or traumatic or periprosthetic or linear or spiral or compression or avulsion or impacted or burst or chance or flexion or supracondylar) adj3 fracture*).ti,ab,kw.
15	((holstein-lewis or jefferson or clay-shoveler or holdsworth or hume or essex-lopresti or galeazzi or colles? or smith* or barton* or monteggia or rolando or bennett* or boxer* or duverney or pilon or bumper or segond or gosselin or toddler* or bosworth or maisonneuve or "le fort" or pott* or lisfranc or jones or march) adj3 fracture*).ti,ab,kw.
16	((skull or mandibular or nasal or cervical or rib? or sternal or shoulder? or arm? or humerus or forearm? or ulnar or radius or distal or scaphoid or pelvic or femoral or patella or crus or tibia? or trimalleolar or calcaneal or bimalleolar) adj3 fracture*).ti,ab,kw.
17	or/8-16
18	7 and 17
19	18 not (humans/ not (humans/ and animals/))
CINAHL	
1	(MH "Antiinflammatory Agents, Non-Steroidal+")
2	TI (non-steroid* N3 (inflammat* or anti-inflammat* or antiinflammat*)) OR AB (non-steroid* N3 (inflammat* or anti-inflammat* or antiinflammat*))
3	TI ((cyclooxygenase or cyclo-oxygenase or COX-2) N1 (inhibit* or attenuat*)) OR AB ((cyclooxygenase or cyclo-oxygenase or COX-2) N1 (inhibit* or attenuat*))
4	TI (adapalene or ampyrone or antipyrine or apazone or aspirin or bufexamac or carprofen or celecoxib or clonixin or curcumin or diclofenac or diflunisal or dipyrone or epirizole or etanercept or etodolac or

	fenoprofen or feprazone or flurbiprofen or ibuprofen or indomethacin or ketoprofen or ketorolac or masoprocol or meclofenamic or mefenamic or mesalamine or naproxen or niflumic or olopatadine or oxyphenbutazone or phenylbutazone or piroxicam or salicylate* or sulfasalazine or sulindac or suprofen or tolmetin) OR AB (adapalene or ampyrone or antipyrine or apazone or aspirin or bufexamac or carprofen or celecoxib or clonixin or curcumin or diclofenac or diflunisal or dipyrone or epirizole or etanercept or etodolac or fenoprofen or feprazone or flurbiprofen or ibuprofen or indomethacin or ketoprofen or ketorolac or masoprocol or meclofenamic or mefenamic or mesalamine or naproxen or niflumic or olopatadine or oxyphenbutazone or phenylbutazone or piroxicam or salicylate* or sulfasalazine or sulindac or suprofen or tolmetin)
5	TI (NSAID? or tactupump or galderma or differin or auralgan or paladin or salicylic or floctafenine or meloxicam or nabumetone or napafenac or oxaprozin or tenoxicam or tiaprofenic) OR AB (NSAID? or tactupump or galderma or differin or auralgan or paladin or salicylic or floctafenine or meloxicam or nabumetone or napafenac or oxaprozin or tenoxicam or tiaprofenic)
6	S1 OR S2 OR S3 OR S4 OR S5
7	(MH "Fractures+")
8	(MH "Bone and Bones+/DE/IN/ME/PA/PP")
9	(MH "Fracture Healing")
10	(MH "Bone Remodeling+")
11	(MH "Bone Density")
12	TI (bone N3 (broken or fracture* or remodel* or regenerat* or density)) OR AB (bone N3 (broken or fracture* or remodel* or regenerat* or density))
13	TI ((fracture? or bone?) N3 heal*) OR AB ((fracture? or bone?) N3 heal*)
14	TI ((displace* or open or closed or comminuted or greenstick or transverse or oblique or buckled or pathologic or stress or hairline or compound or traumatic or periprosthetic or linear or spiral or compression or avulsion or impacted or burst or chance or flexion or supracondylar) N3 fracture*) OR AB ((displace* or open or closed or comminuted or greenstick or transverse or oblique or buckled or pathologic or stress or hairline or compound or traumatic or periprosthetic or linear or spiral or compression or avulsion or impacted or burst or chance or flexion or supracondylar) N3 fracture*)
15	TI ((holstein-lewis or jefferson or clay-shoveler or holdsworth or hume or essex-lopresti or galeazzi or colles? or smith* or barton* or monteggia or rolando or bennett* or boxer* or duverney or pilon or bumper or segond or gosselin or toddler* or bosworth or maisonneuve or "le fort" or pott* or lisfranc or jones or march) N3 fracture*) OR AB ((holstein-lewis or jefferson or clay-shoveler or holdsworth or hume or essex-lopresti or galeazzi or colles? or smith* or barton* or monteggia or rolando or bennett* or boxer* or duverney or pilon or bumper or segond or gosselin or toddler* or bosworth or maisonneuve or "le fort" or pott* or lisfranc or jones or march) N3 fracture*)
16	TI ((skull or mandibular or nasal or cervical or rib? or sternal or shoulder? or arm? or humerus or forearm? or ulnar or radius or distal or scaphoid or pelvic or femoral or patella or crus or tibia? or trimalleolar or calcaneal or bimalleolar) N3 fracture*) OR AB ((skull or mandibular or nasal or cervical or rib? or sternal or shoulder? or arm? or humerus or forearm? or ulnar or radius or distal or scaphoid or pelvic or femoral or patella or crus or tibia? or trimalleolar or calcaneal or bimalleolar) N3 fracture*)
17	S7 OR S8 OR S9 OR S10 OR S11 OR S12 OR S13 OR S14 OR S15 OR S16
18	S6 AND S17
Scopus	
(INDEXTERMS ("Antiinflammatory Agents, Non-Steroidal") OR TITLE-ABS-KEY (non-steroid* W/3 (inflammat* OR anti-inflammat* OR antiinflammat*)) OR TITLE-ABS-KEY ((cyclooxygenase OR cyclo-oxygenase OR cox-2) W/1 (inhibit* OR attenuat*)) OR TITLE-ABS-KEY (adapalene OR ampyrone OR antipyrine OR apazone OR aspirin OR bufexamac OR carprofen OR celecoxib OR clonixin OR curcumin OR diclofenac OR diflunisal OR dipyrone OR epirizole OR etanercept OR etodolac OR fenoprofen	

OR feprazone OR flurbiprofen OR ibuprofen OR indomethacin OR ketoprofen OR ketorolac OR masoprocol OR meclofenamic OR mefenamic OR mesalamine OR naproxen OR niflumic OR olopatadine OR oxyphenbutazone OR phenylbutazone OR piroxicam OR salicylate* OR sulfasalazine OR sulindac OR suprofen OR tolmetin) OR TITLE-ABS-KEY (
nsaid? OR tactupump OR galderma OR differin OR auralgan OR paladin OR salicylic OR floctafenine OR meloxicam OR nabumetone OR napafenac OR oxaprozin OR tenoxicam OR tiaprofenic)) AND (INDEXTERMS ("Fractures, Bone") OR INDEXTERMS ("Bone and Bones") OR INDEXTERMS ("Fracture Healing") OR INDEXTERMS ("Bone Remodeling") OR INDEXTERMS ("Bone Density") OR TITLE-ABS-KEY (bone W/3 (
broken OR fracture* OR remodel* OR regenerat* OR density)) OR TITLE-ABS-KEY ((fracture? OR bone?) W/3 heal*) OR TITLE-ABS-KEY ((
displace* OR open OR closed OR comminuted OR greenstick OR transverse OR oblique OR buckled OR pathologic OR stress OR hairline OR compound OR traumatic OR periprosthetic OR linear OR spiral OR compression OR avulsion OR impacted OR burst OR chance OR flexion OR supracondylar) W/3 fracture*) OR TITLE-ABS-KEY ((
holstein-lewis OR jefferson OR clay-shoveler OR holdsworth OR hume OR essex-lopresti OR galeazzi OR colles? OR smith* OR barton* OR monteggia OR rolando OR bennett* OR boxer* OR duverney OR pilon OR bumper OR segond OR gosselin OR toddler* OR bosworth OR maisonneuve OR "le fort" OR pott* OR lisfranc OR jones OR march) W/3 fracture*) OR TITLE-ABS-KEY ((
skull OR mandibular OR nasal OR cervical OR rib? OR sternal OR shoulder? OR arm? OR humerus OR forearm? OR ulnar OR radius OR distal OR scaphoid OR pelvic OR femoral OR patella OR crus OR tibia? OR trimalleolar OR calcaneal OR bimalleolar) W/3 fracture*)) AND NOT DBCOLL (medl)

Biosis (Ovid)	
1	(non-steroid* adj3 (inflammat* or anti-inflammat* or antiinflammat*)).ti,ab,kf.
2	((cyclooxygenase or cyclo-oxygenase or COX-2) adj1 (inhibit* or attenuat*)).ti,ab,kf.
3	(adapalene or ampyrone or antipyrine or apazone or aspirin or bufexamac or carprofen or celecoxib or clonixin or curcumin or diclofenac or diflunisal or dipyrone or epirizole or etanercept or etodolac or fenoprofen or feprazone or flurbiprofen or ibuprofen or indomethacin or ketoprofen or ketorolac or masoprocol or meclofenamic or mefenamic or mesalamine or naproxen or niflumic or olopatadine or oxyphenbutazone or phenylbutazone or piroxicam or salicylate* or sulfasalazine or sulindac or suprofen or tolmetin).ti,ab,kf.
4	(NSAID? or tactupump or galderma or differin or auralgan or paladin or salicylic or floctafenine or meloxicam or nabumetone or napafenac or oxaprozin or tenoxicam or tiaprofenic).ti,ab,kf.
5	or/1-5
6	(bone adj3 (broken or fracture* or remodel* or regenerat* or density)).ti,ab,kf.
7	((fracture? or bone?) adj3 heal*).ti,ab,kf.
8	((displace* or open or closed or comminuted or greenstick or transverse or oblique or buckled or pathologic or stress or hairline or compound or traumatic or periprosthetic or linear or spiral or compression or avulsion or impacted or burst or chance or flexion or supracondylar) adj3 fracture*).ti,ab,kf.
9	((holstein-lewis or jefferson or clay-shoveler or holdsworth or hume or essex-lopresti or galeazzi or colles? or smith* or barton* or monteggia or rolando or bennett* or boxer* or duverney or pilon or bumper or segond or gosselin or toddler* or bosworth or maisonneuve or "le fort" or pott* or lisfranc or jones or march) adj3 fracture*).ti,ab,kf.
10	((skull or mandibular or nasal or cervical or rib? or sternal or shoulder? or arm? or humerus or forearm? or ulnar or radius or distal or scaphoid or pelvic or femoral or patella or crus or tibia? or trimalleolar or calcaneal or bimalleolar) adj3 fracture*).ti,ab,kf.
11	or/7-16
12	5 and 11

Table 12-3S: Studies characteristics

Study	Year	Species	Age	Weight	Sex	Type of bone	Type of NSAID	Dose	Duration	Primary outcome measures	Time point	Other measurement	Time point
Akman et al(2)	2002	Rats	20	270	M	Tibia	NS-COX(Dicl.)	1,2	10	NO	NA	Clin, Radiog, Hist-scores(Huo)	14,28.42
Allen et al(3)	1980	Rats	6	NA	M	Other	NS-COX(Ind.,Asp.)	2,4,100,200,300	21	NO	NA	Hist-Scores	21
Altman et al(4)	1995	Rats	retired	375	F	Femur	NS-COX(ind.,ibu.)	1,30	4,12	3PMB-FR	14,28,48,57,84	Hist-Scores(Huo)	14,28,48,57,84
Beck et al(5)	2003	Rats	NA	325	M	Tibia	NS-COX(Dicl.)	5	7,21	3PMB-F,3PMB-Stiff	21	BD-µ-CT	21
Bergenstock et al (6)	2005	Rats	NA	301	F	Femur	COX-2(Celec.)	3,6,60,300	10	3PMB-F,3PMB-Stiff	56	Radiog.(scores), Non-union ratio, Hist.	56
Brown et al(7)	2004	Rats	NA	300	M	Femur	NS-COX(Ind.), COX-2(Celec.)	1,3	28,56,84	3PMB-F,3PMB-Stiff	28,56,84	Radiog, Hist-grades	28,56,84
Cappello et al(8)	2013	Rats	3	NA	M	Tibia	NS-COX(Ket.)	5	7,14,21	4PMB-F	21	Hist-description	7,14,21
Dimmen et al(9)	2008	Rats	adult	213	F	Tibia	COX-2(Par.)	10	7	3PMB-F,3PMB-Stiff	6	DEXA-BD	14,21,42
Dimmen et al(10)	2009	Rats	NA	226.5	F	Tibia	NS-COX(Ind.),COX-2(Par.)	1.625,1	7	3PMB-F,3PMB-Stiff	21	DEXA-BD	14,21
Lack et al(11)	2013	Rabbit	NA	NA	NA	Ulna	NS-COX(Ind.,Aspirin)	10,10	56	3PMB-F	56	Radiog.,Hist.	14,28,42,56
More et al(12)	1989	Rabbit	Adolescent	260	F	Tibia	NS-COX(Flunixin,Piroxicam)	1.1,0.2	21	3PMB-F	21	Ankle stiffness	Not clear
Endo et al(13)	2005	Rats	12	250	NA	Femur	COX-2(Etodolac)	20	7	3PMB-F,3PMB-Stiff	21	Radiog.(scores)	7,14,21
Endo et al(14)	2002	Rats	12	250	F	Femur	COX-2(Etodolac)	20	21	3PMB-F,3PMB-Stiff	21	Radiog.(scores)	7,14,21
Gerstenfeld et al(15)	2007	Rats	NA	449	M	Femur	NS-COX(Ket.), COX-2(Valdecoxib)	4,5	7,21	3PMB-F,3PMB-Stiff	21,35	Hist-mineralization, PGE2 level	21,35
Gerstenfeld et al(16)	2003	Rats	10	430	M	Femur	NS-COX(Ket.), COX-2(Valdecoxib)	4,5	21,35	3PMB-F,3PMB-Stiff	21,35	Hist.	21,35
Giordano et al(17)	2003	Rats		100	M	Tibia	COX-2(Tenoxicam)	20	7,14,28	NO	NA	Hist.scores(Allen	3,7,14,28

Hak et al(18)	2011	Rabbit	48	300	F	Tibia	COX-2(Rof.)	12.5	28	3PMB-F,3PMB-Stiff,3PMB-WF	28	Hist., COX-1 & COX-2 mRNA level	28
Herbenick et al (19)	2008	Rats	NA	350	M	Femur	COX-2(Celec.)	3.2	14,28,56,84	3PMB-F	14,28,56,84	NO	
Hogevold et al (20)	1992	Rats	NA	338	M	Femur	NS-COX(Ind.)	0.2,0.2	3	3PMB-F,3PMB-Stiff,3PMB-WF	6	NO	
Huo et al(21)	1991	Rats	Mature	240	F	Femur	NS-COX(Ibu.)	30	5days started after 3 days	3PMB-F,3PMB-Stiff	14,28,42,56,84	Hist., BV, (Huo grades)	14,28,42,84
Inal et al(22)	2014	Rats	NA	250	M	Fibula	NS-COX(Dicl.,Dexketoprofen) COX-2(Melo.)	1,0.98,0.2	10	NO	NA	Hist.Scores (Huo)	28
Keller et al(23)	1987	Rabbit	adult	430	NA	Tibia	NS-COX(Ind.)	10	14,42	3PMB-F	14,42	Hist.Bone tissue	14,42
Krischak et al(24)	2007	Rats	NA	300	M	Femur	MS-COX(Dicl.)	5	10	NO	NA	Hist.Bone density (No.osteoblasts), Radiog.	10
Krischak et al(25)	2007	Rats	NA	300	M	Tibia	NS-COX(Dicl.)	5	7,21	NO	NA	Hist.(tissue type, bone, cartilage, and fibrous)	21
Li et al(26)	2013	Rats	11	276	F	Femur	COX-2(Celec.)	21	1,4	3PMB-F,3PMB-Stiff,3PMB-WF	42	Histo-Bone density	28
Matsumoto et al (27)	2008	Rats	8	250	M	Tibia	NS-COX(Ketoprofen), COX-2(Celec.)	1,4	3 or till scarifice	NO	NA	Hist.Bone area, Immunohistochemistry	7,14,21
Mullis et al(28)	2006	Mice	10	25	M	Tibia	NS-COX(Ket.,Ind.), COX-2(Celec.,Rof.)	2,2,10/50, 1/5)	28,56,84	3PMB-F,3PMB-Stiff	28,56,84	Hist.(cartilage area, Trap), Biochemical	28,56,84
Murnaghan et al (29)	2006	Mice	16	45.28	M	Femur	COX-2(Rof.)	5	24,32	3PMB-F,3PMB-Stiff	24,32	Histo.Scores(callus size, Fibrous, cartilage,bone), Radiog.(BD)	24,32
Reikeraas & Engebretsen(30)	1998	Rats	NA	300	M	Femur	NS-COX(Ket.,Ind.)	1,2	42	3PMB-F,3PMB-Stiff,3PMB-WF	42	Radiog.	42

Sandberg& Aspenberg(31)	2015	Mice	10	NA	M	Femur	NS-COX(Ind.)	2	7	3PMB-F,3PMB-Stiff	17	µ-CT.(BV,TV,BV/Tv,T.Th.,T.SP)	17
Sassioto et al(32)	2006	Rats	NA	355	M	Femur	NS-COX(Dicl.)	5	NA	NO	NA	Hist.(Examination for fibrous, cartilage & bone tissues)	7,14,21
Sevimli et al(33)	2013	Rats	11.6	190	M	Tibia	NS-COX(Dexketoprofen)	5	14,21,24	3PMB-F	24	Hist.Scores(Huo),Radiog.Scores	14,28,56
Simon et al(34)	2002	Rats	NA	584	M	Femur	NS-COX(Ind.), COX-2(Celec.,Rof.)	1,3,4	28,42,56	3PMB-F,3PMB-Stiff,3PMB-WF	28,42,56	Hist.(MT,Callus Size)	28,42,56
Simon et al(35)	2007	Rats	NA	272	F	Femur	COX-2(Celec.)	2,4,8	Different effect after 15 days and effect of 4mg/kg in different time points	3PMB-F,3PMB-Stiff	15,5,10,15,21,28	Radiog.scores	Different effect after 15 days and effect of 4mg/kg in different time points
Singh et al (36)	2011	Rabbit	adult	240	NA	Femur	COX-2(Etoricoxib)	3	28, 56, 84	3PMB-F,3PMB-Stiff	28,56,84	Hist. scores, Radiog. scores, Morphological	28,56,84
Spiro et al(37)	2010	Mice	12	NA	F	Femur	NS-COX(Dicl.)	5	20	3PMB-F	20	Hist.(MT,OSB & OSC number & Surface), CT(BV,TV,BV/TV,T.Th,T.Sp)	20
Tan et al(38)	2009	Rabbit	NA	350	M	Other	NS-COX(Ibu.), COX-2(Rof.)	50,12.5	28	3PMB-F,3PMB-Stiff	42, 84	Hist.(MT, callus Size, Cartilage)	21,42
Tiseo et al(39)	2006	Rats	NA	341	NA	Femur	NS-COX(Dicl.), COX-2(Rof.)	3,1	14,28	NO	NA	Hist.(callus area, Bone area,bone new formation area), Radiog.	14,28
Tornkvist et al(40)	1984	Rabbit	16	210	NA	Femur	NS-COX(Ibu.,Ind.)	15,10	56	3PMB-F	56	NO	NA
Utvag et al(41)	2010	Rats	113	350	M	Tibia	NS-COX(Dicl.), COX-2(Par.)	1,2	7	3PMB-F,3PMB-Stiff,3PMB-WF	30	DEXA-BD	30
Bissinger et al 2016(42)	2016	Rats	16	500	M	Femur	NS-COX(Dicl.)	5,0.5	21	3PMB-F,3PMB-Stiff	21	µ-CT(BV,TV,BV/TV,T.Th,T.S)	21

Bo et al 1976(43)	1976	Rats	Adolescent	187	M	Femur	NS-COX(Ind.)	2	6,9,12,18,24	3PMB-F,3PMB-Stiff	6,9,12,18,24	Hist.(area of mineralization), Plasma level of Indomethacin	21
Ochi et al 2011(44)	2011	Dogs	11m	NA	F	Tibia	NS-COX(Carprofen)	2.2	120	3PMB-F,3PMB-Stiff	120	Hist.(callus area,Cartilage), Radiog.	120
Karachalios et al (45)	2007	Rabbit	12	340	M	Ulna	NS-COX(Ind.), COX-2(Melo.,Rof.)	2,0.3, 0.5,2.5	5	3PMB-F,3PMB-Stiff.	42	Hist.(histomorpho metrics- BC,OCC)	42
Sudman & Bang(46)	1979	Rabbit	18	200	F	Radius	NS-COX(Ind.)	5,10	14,28	NO	NA	Hist.(Flourochrom label for harvesian system), Indomethcin Plasma level	Not Clear

NA-not available; NO-not measured; M-male; F-female; NS-COX-nonselective cyclooxygenase; COX-2-selective cyclooxygenase-2; 3PMB-F-3 points mechanical bending (force to fracture); 3PMB-stiff.- 3 points mechanical bending (stiffness); 3PMB-WF-3 points mechanical bending (work to failure); µ-CT- micro-computertomographic; MT-mineralized tissue; BV-bone volume; BV/TV-bone volume/ tissue volume; T.Th.-trabeculae thickness; T.Sp-trabeculae space; OSB-osteoblast number; OSC-osteclast number

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13 Appendix II- Supplementary materials of manuscript II

Supplementary information for material and Methods of manuscript II

Guarding behaviour test (GBT)

Guarding behaviour test was done according to already stablished protocol (1). Each mouse was placed individually on an elevated stainless-steel mesh. Both paws (of the fractured and non-fractured legs) were carefully observed for one-minute periods every five minutes over one hour. A score of 0, 1, or 2 was given according to the postural position of each paw in the one-minute scoring periods. Score 0 was given if the paw skin was blanched or distorted by the mesh (indicating weight bearing), while score 2 meant that the paw was entirely off the floor. If the paw touched the floor without blanching or distorting, then a score of one was assigned. After the 1-hour session, the sum of the 12 scores (0–24) given during the session was calculated for each paw. Subsequently, the scores of the fractured and non-fractured legs were compared between different experimental groups.

Weight-bearing test (WBT)

Weight bearing test was done to test the recovery of treated limb according to an already established protocol (2). The relative distribution of weight the animal bear on the injured, and uninjured legs was determined by an in-capacitance meter (IITC.inc, CA, US). Each mouse was positioned in an angled chamber so that each hind paw rests on a separate weighting plate. The weight exerted by each hind leg was measured for 5 seconds and then averaged. The change in paw weight distribution was calculated by determining the difference in weight (g) between the left (control) and right (fractured) legs. This difference had been used as a pain index in the fractured leg.

Microcomputed tomography (µCT) assessment

Ex-vivo scans of 14-day harvested tibiae were conducted using a micro-CT (Skyscan 1172; Bruker-microCT, Kontich, Belgium). Scans were taken with a 5.88 mm pixel size, at scanner voltage and

current set to 59 kV and 167 lA, respectively. 3D reconstructions were created using the (CT-Analyser; Bruker micro-CT, Kontich, Belgium) software to measure the callus size, total volume (TV), and bone volume (BV). The bone volume fraction (BV/TV), and the trabecular number and thickness were calculated for the volume of interest, which encompasses the fracture callus within a 1.5 mm (255 slices) vertical range, centred on the osteotomy site. The region of interest (ROI) for each section was selected as the outer boundary of the fracture callus, excluding the fibula. A binary threshold gray level of 68/255, corresponding to the murine trabecular bone, was used to segment mineralized bone from soft tissue (3-6) (Table 13-5S).

Biomechanical testing

The biomechanical properties of the fracture site were tested according to a previous protocol (7). A three-point bending test performed using a Mach-1a mechanical testing machine (Biomomentum ®, Laval, Quebec). The distance between the supports with the bending fixture was 10 mm, and the diameter of the supports and loading nose was 0.25 mm. The amchine applied downword load to the fracture site of the bone at a rate of 0.016 mm/second until failure. A load-displacement curve generated using Mach-1 software (Tempe, Arizona); this was used to determine three parameters: stiffness (N/mm), ultimate force (N), and work to failure (N*mm).

Histological analysis

We dehydrated the bone defect samples (right tibiae) in ascending concentrations of ethanol (70–95%) and embedded them in methyl methacrylate at the Bone Biology Lab, McGill University. After polymerization, three subsequent 6-µm-thick sections, crossing through the middle of the defect, were obtained from each sample and stained with either tartrate-resistant acid phosphatase (TRAP), Masson's-trichrome or Von-Kossa stain to assess osteoclasts, collagen, and mineralization, respectively. For each sample, we analyzed one histological section per stain. Images of the histological slides were produced using an optical microscope (Zeiss-Microscopy, Jena, Germany).

We defined the region of interest for osteoclast, collagen and mineralized tissue histomorphometry as the area of the histological section enclosed by the cortical margins and the borders of the healing callus of the fracture site. Osteoclasts were calculated using ZEN-2012-SP2 imaging software (Zeiss-Microscopy, Jena, Germany), and the data was demonstrated as osteoclasts number per square millimetre of mineralized tissue (OC/mm2). We analyzed the percentages of mineralized tissue and collagen in the region of interest of the healing site using ImageJ v1.45 (Wayne Rasband; NIH, Bethesda, MD, USA) and documented as mineralized tissue percent (MT%). Histomorphometric analysis performed by an operator, who was blinded to group allocation (8, 9).

Cytokines profile for blood serum

Blood serum was collected from each mouse at day three after the surgery and frozen at -80° in separate vials until further analysis. For cytokine analysis, the serum samples were thawed at room temperature. A multiplex inflammatory cytokine kit (Mouse Cytokine Magnetic 20-Plex Panel-Thermofisher Scientific, Waltham, MA) and the Luminex 100/200 Multiplexing Instrument (Luminex Corp., Austin, TX) was used to analyze the inflammatory cytokine profile of each serum sample. The Mouse Cytokine Magnetic 20-Plex kit was used for bead assays of FGF basic, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p40/p70), IL-13, IL-17, IP-10, KC, MCP-1, MIG, MIP-1α, TNF-α, and VEGF. These assays were performed following the manufacturer's protocol. The Luminex®100/200 System and xPONENT instrumentation software were used for measurements and analysis (10, 11).

RNA isolation and Whole-Transcript Expression Analysis

The central one-third of the fractured tibia including all the callous and hematoma was harvested on day three post-fracture from the experimental groups and stored in Alloprotect (Qiagen, Toronto, Ontario). The total RNA was isolated from bone tissue using QAlzol (Qiagen, Toronto, Ontario) and then purified using RNeasy Lipid Tissue Mini Kit columns (Qiagen, Toronto, Ontario)

following the manufacturer's instructions. The extracted total RNA sent for Whole-Transcript Expression Analysis. Total RNA was quantified using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc., Waltham, MA) and its integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies, Waltham, MA) (Fig.S1a,b). Sense-strand cDNA synthesized from 100 ng of total RNA, and fragmentation and labelling were performed to produce ssDNA with the Affymetrix GeneChip® WT Terminal Labeling Kit (Thermofisher, Waltham, MA) according to the manufacturer's instructions. After fragmentation and labelling, 3.5µg DNA target was hybridized on GeneChip® Mouse Gene 2.0 ST (Affymetrix, Waltham, MA) and was incubated at 450C in the Genechip® Hybridization oven 640 (Affymetrix, Waltham, MA) for 17 hours at 60 rpm. The GeneChips then were washed in a GeneChips® Fluidics Station 450 (Affymetrix) using Affymetrix Hybridization Wash and Stain kit according to the manufacturer's instructions (Affymetrix). The microarrays finally were scanned on a GeneChip® scanner 3000 (Affymetrix). The data were normalized, and the analysis was only included the identified units within the samples. The analysis was conducted using GeneSpring GX 10 software (Agilent, Santa-Clara, CA, United States). To identify genes differentially expressed between groups of mice that received NSAID during active versus resting time and control groups, we used unpaired Student's t-tests to compare between groups. The genes that presented an up- or down-regulation with p-value < 0.05 were identified. To avoid loss of substantial numbers of true positive genes, no correction was performed for multiple testing.

Using the Gene Ontology (GO) database (PANTHER Classification version 11), the PANTHER Overrepresentation Test was performed on the list of genes that were differentially and significantly expressed (as described above) to identify the functional biological processes involved in the genes. Specifically, this binomial test determines whether there is a statistical overrepresentation or underrepresentation of the genes that expressed differently between the active versus resting time group relative to the reference list in the GO ontology database for the mice species (a full list is

published in the Gene Expression Omnibus (GEO) data repository, available upon request). Using the PANTHER Classification System, a 'gene ontology' classification of the genes/expressed sequence tags (ESTs) that are significantly ($p < 0.05$) up- or downregulated was performed to compare between different experimental groups. We classified differentially expressed genes into various biological processes to assess their functional significance, then calculated the proportional distribution of genes in each process.

The list of genes that significantly differentiated between active versus resting time groups were employed to identify significantly activated pathways by comparing their functional annotations according to the PANTHER Classification System (www.pantherdb.org) with the whole mouse genome (data updated to NCBI's January 10, 2011 release) by the binomial distribution function (12).

Table 13-1S: Phases during secondary fracture healing.

Timing of cellular and expression of signalling molecules during mice fracture healing (16-20).

Day	Event	Molecular signalling	
		Cells	**Signals/Cytokine**
1	**Hematoma formation and initiation of acute inflammation** (Activation and recruitment of Proinflammtory cells and cytokines)	Macrophages, neutrophils and attraction of mesenchymal stem cells	***IL-1, IL-6, IL-8, IL-11, TNF-α, CCL2, CCL7, IP-10*** (from inflammatory cells-Monocytes /macrophage) ***PDGF, TFG-β*** (from degranulation platelets) ***BMP-2*** expression ***GDF-8*** and osteoprotegerin expression
3	**Fibrous tissue** (Angiogenesis)	Mesenchymal stem cells, osteoblast start to differentiate in intramembranous bone	Expression of: ***CCL3***, ***CCL11, IL-10, HIF1 α protein, HMOX, VEGF and GLUT1, TGF-β2, β3, GDF-10, BMP-5, -6 and RANKL and M-CSF, MIF (decreased for 2wks)*** Decline of cytokines: ***CCL7, IL-6, IL-1*** Induction of ***Angiopoietin-1(angio***
7	**Soft and hard callus formation** (Endochondral ossification)	Intramembranous ossification's cell proliferation (peaks between 7-9 days). Maturation of chondrocytes (days 9-14)	Expression of ***CXCL12*** The peak of ***TGF- β2, β3 &*** osteoprotegerin Expression of ***GDF-5 and*** probably ***GDF-1***
14	**Cartilage resorption and Active osteogenesis**	Osteoblastic and osteoclastic cells activities, along with new mesenchymal cells	Decreased levels of expression for ***TGF-b2, GDF-5, and*** probably ***GDF-1*** Expression of ***BMP-3, -4, -7, and -8*** Expression of ***VEGF and RANKL, M-CSF*** Peak of ***IL-1 and TNF-a*** expression that continues during bone remodelling
21	**Remodelling**	Continuous osteoblastic and osteoclastic activities	Second peak of ***IL-1, IL-6, and TNF-a*** expression which continues during bone remodelling

Table 13-2S: Circadian rhythm of immune cells and cytokines expression during the day in various species (Human and Mice).

Cytokines/ Chemokines ligands or receptors/	Role in inflammation, and bone healing	Circadian Rhythm	Reference
IL-4	Th2 cytokines that are associated with macrophage M1-M2 polarization. Expresses at 3-4 days after fracture associated with anti-inflammatory macrophage trait	H-ZT18	(21) (17)
VEGF	Secreted by endothelial cells, induce angiogenesis and growth. Expresses at the end of hematoma formation when	M-serum Peak during the dark phase	(22)

	the hypoxia state start (around 4 to 14 days after fracture)	and lower levels at light phase (ZT2 and ZT14)	
IL-6	IL-6 is the cytokine largely responsible for inducing the synthesis of the acute phase proteins C reactive protein and serum amyloid protein A (SAA) and is one of the major cytokines involved in bone resorption. Highly expressed in day 1 and with presence of inflammation expressed at day 7 and 14	H-Morning 7:30 Healthy and RA patients at 4:00 am	(23), (24) (18)
IL-10	Healing in fetus(25), Expressed at day 4-7 after fracture	H-Morning 7:30 with second peak at 13:30	(23)
TNF-a and its receptors (p55 and p66)	expressed in day 1 and later in day 21 and 28, the depressed level at day 7	H-S-Morning 7:30 6:00 am in healthy and RA patients	(23) (18)
MMP-9	Potent degenerative enzyme	H-Tears-Increase 200-fold on awakens	(26)
IL-1B	One of the most potent osteoclast-activating factors within the human organism and is thus believed to play an important role in periodontal tissue breakdown, IL-1 peak ion the 3rd day after fracture, expressed in day 1 and later in day 21 and 28, depressed level at day 7	H-GCF-Periodontal healthy subjects-lowest morning, highest evening (melatonin can affect)	(27) (28)
Osteoprogetron (OPG) and its receptor (RNKL)	Peaks in the fracture site after 24hours and at the peak of cartilage formation phase day 7, while RANKL osteoprotegerin was seen on day 3 and 14 like MCSF		(18)
IL-1R1 and receptors	Expresse on day 1 and 3 post fracture		(18)
IL-5	Recruitment and activation of the OPG and inhibition of osteoclast activity		(29)
Macrophage inhibitory factor (MIF)	Expressed at day 4 of fracture and decreased gradually for two weeks. Counter-regulator of glucocorticoid action. Induced immunosuppression and glucocorticoid Induced proinflammatory cytokine inhibition. 8% of macrophage cells and their secretion is under local circadian control-IL-6 or TNF-a	H- peaks 6:00-9:00 am Nadir: 00:00 to 3:00	(30), (31) (32) (33)
CXchemokines ligands CXCL12	Induced recruitment and retention of haemopoietic stem cells and macrophage cells and mature immune cells, Upregulate during the acute phase of bone healing	Down regulate at the beginning of the resting phase to allow release of the cells in the blood (ZT23, ZT1, ZT24)	(17)
CXCR4	Receptor of the CCL12 on the cells and CD4 and CD8 and T cells.	Peak during the resting phase	(17)

	Associated with the recruitment of BM-MSCs to the fracture site		
P-selectin, E-selectin, VCAM-1 ICAM-1, Ccl2	Initiation of the inflammatory phase of the endochondral ossification healing. Express in day1 – day3	M-bone marrow – (ZT13)	(17)
INF	Role in initiation and activation of macrophage polarization from M0 to M1. Expresses in day 3	Natural killer - M – (ZT 14-24)	(17)
TLR9 Toll-like receptor 9	Modulates the inflammatory response during bone helaing and may affect the osteoclastogenesis through its affect on osteoclasts cells or osteoblasts. Its subunit of TLR family	M-ZT19-7	(34)
Neutrophils, lymphocyte, monocyte, eosinophils	Recruited to the fracture site for secretion and initiation the inflammatory phase, Highly expressed at day 1	M-CT17 (during active phase)	(35)
Macrophages	Recruited at the beginning of the inflammatory phase at the fracture site (role in secretion of different proinflammatory and anti-inflammatory cytokines)	M-CT0-12 (during resting phase)	(33)
B-cells and T-cells	During later stage of inflammatory phase, B-cells and T-cells are involved in suppression of the pro-inflammatory signals, and able to induce anti-inflammatory functions from mesenchymal stem cells and has been shown to induce osteogenic differentiation and activity	M-ZT5-13	(36)

Human, M-mouse, CT: actual circadian time in hours (e.g. CT6 = 6AM); ZT: Zeitgeber time: time after the onset of light with lights on at ZT0/24 and off at ZT12ZT 0-light on, ZT12-Light off

Table 13-3S: The percentage distribution of biological process ontologies identified for upregulated and downregulated between active time administration of NSAID group versus resting time administration of NSAID after fracture surgery on day 3.

Biological processes ontologies	**N genes (%) Significantly upregulated genes**	**N genes (%) Significantly downregulated genes**
Biological regulation	5(9.6)	28(25.5)
Cellular component organization and biogenesis	5(9.6)	19(17.3)
Biological adhesion	-	5(4.5)
Cellular process	18(34.6)	47(42.7)
Developmental process	1(1.9)	7(6.4)
Immune system process	1(1.9)	21(19.1)
Localization	5(9.6)	24(21.8)
Locomotion	1(1.9)	2(1.8)
Metabolic process	17(32.7)	42(38.2)
Multicellular organismal process	5(9.6)	18(16.4)
Reproduction	1(1.9)	-
Response to stimulus	5(9.6)	25(22.7)
	64	**238**

Statistically significant genes at least 1.5-fold change ($p<0.05$), N gene hits against total number of genes

Figure 13-1S: The percentage distribution of biological process ontologies

Biological processes identified for statistically significant genes ($p < 0.05$) differentially upregulated (a) and downregulated (b) between group received NSAID upon active time as compared to that in the group received NSAID at a resting time at day three post-surgery.

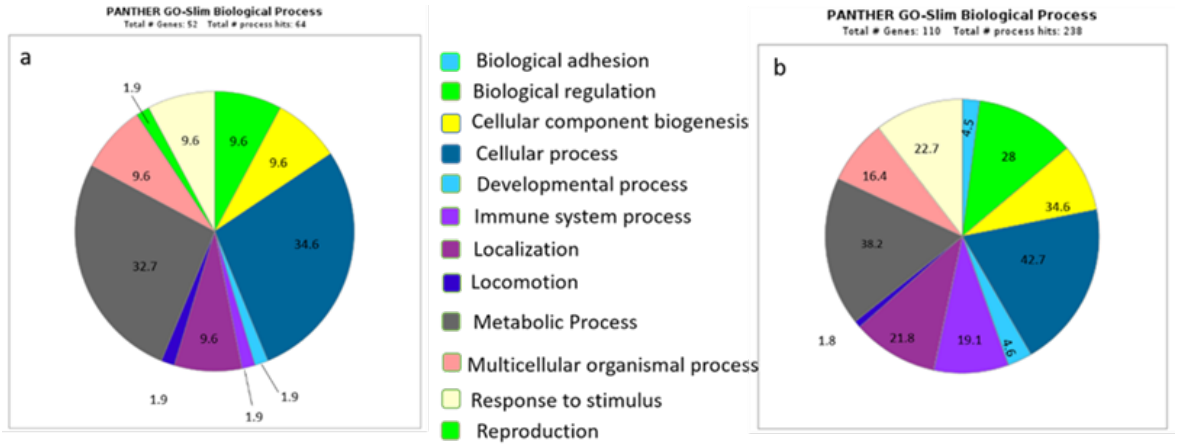


Table 13-4S: Examples of expressed genes of RNA sequencing analysis in the healing callus.

Genes of known function in bone healing after 3 days of NSAID administration at active time in comparison to resting time, p-value < 0.05.

Gene	Cells expresses the gen	Role in bone healing	Reference
Examples of upregulated genes			
CCL3 (Chemokine (C-C motif) ligand 3) Macrophage inflammatory protein	Macrophage	Contribute in the recruitment of mesenchymal stem cells and their differentiation	(17)
CCL12 (Chemokine (C-C motif) ligand 12) monocyte chemotactic protein	Macrophage	Contributes in the recruitment of mesenchymal stem cells and their differentiation	(17) (37)
FGFbp Fibroblast growth factor-binding protein	Fibroblast	Important in the endothelial cellular proliferation	
Retnla/FIZZ1 Resistin-like molecule alpha	Macrophage	Polarization from M1 to M2 phenotype	(37)
IL-4R1/t6N Interleukine-4 receptor subunit-1	Macrophage	Important in activation of M1 to M2 polarization	(37)
Lzic	bone marrow mesenchymal stem cell	Accelerates the degradation of phospho-β-catenin, resulting in an increased level of WNT signaling, a recognized pathway in osteogenesis	(38)
Examples of downregulated genes			
IL-18 Interleukin-18	Macrophage	Overproduction of IL-18 stimulates IFN-γ production and suppresses IL-4 in vivo, resulting in cortical thinning and decreased bone volume in a mouse model	(39)
IL-6rα Interleukin-6 receptor subunit α	Leukocytes (Neutrophils-Pain receptor)	Blockade of IL-6 ra decrease pain and improve improves compromised fracture healing	(40)
STAT1 Signal transducer and activator of transcription 1	Transcription factor that mediate IFNY, IL-6 and IL-12 and many pro inflammatories after acute insult (like a fracture).	Inhibition of Stat1 showed to accelerate bone healing, STAT1 as a crucial negative regulator for both osteoblast differentiation and osteoclastogenesis, and also suggest that inhibition of STAT1 activity may be beneficial for the treatment of skeletal fracture	(41)
IL-9 Interleukine-9	Macrophage and T helper cells	Increased level of IL-9 in the fracture haematoma of delayed bone healing	

Figure 13-2S: Representative Bioanalyzer electropherogram profile of the RNAs contained in the healing callus after open tibia fracture surgery and three days of administration NSAID either during the resting time (a) or during activity time (b).

The electrogram show the size distribution in nucleotides (nt) and fluorescent intensity (FU) of total RNA. The most dominant peaks are 18S and 28S. Associated gel image shown alongside the plot.

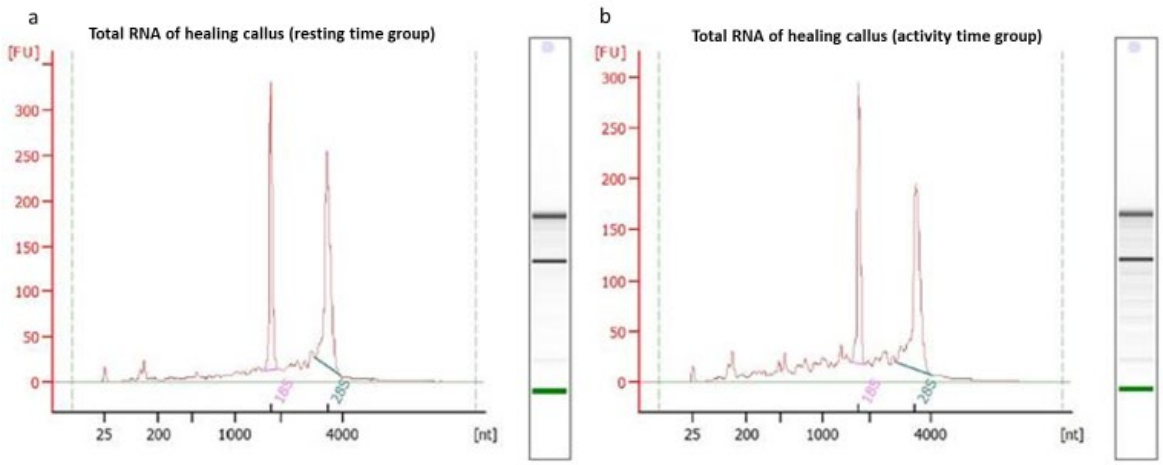
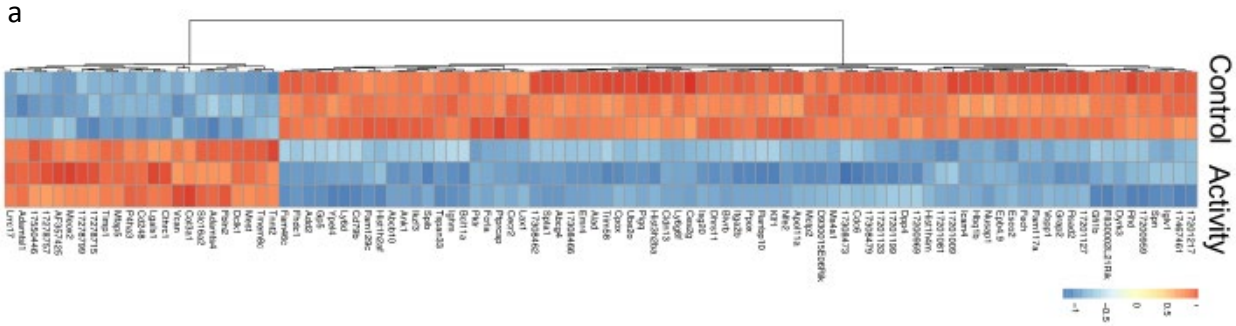


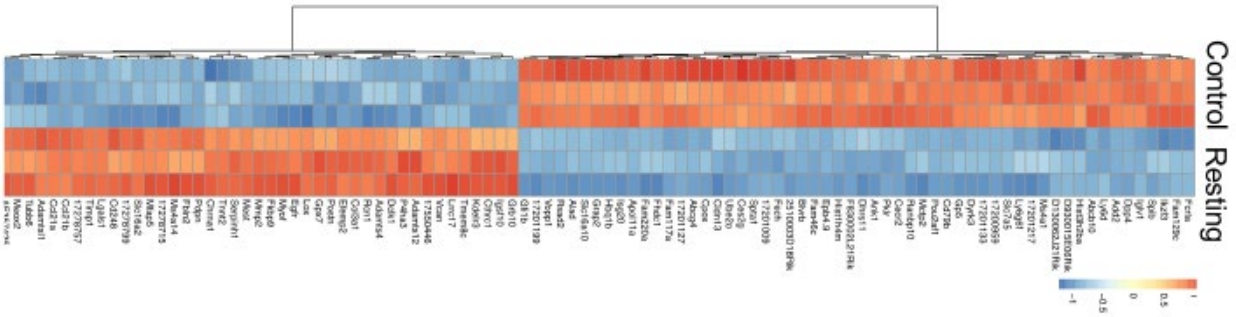
Figure 13-3S: Gene expression heat map for the top 100 genes that showed at least 1.5-fold difference (P<0.05) upregulated (red) or downregulated (blue) at day three after fracture surgery between group receiving NSAID at activity time compared to control (a) and t those receiving NSAID at resting time compared to control (b).

Gene expression profile of bone healing at day three after fracture surgery between group received NSAID upon active time as compared to that in group received NSAID at resting time. (c) Comparison of common and distinct genes of fractured healing callus after NSAID administration for three days either during activity time or at resting time. Fold-change (FC) vs. FC plot of activity time vs. control (without surgery) on the x-axis and resting time vs. control on the y-axis highlighting common significantly expressed genes (adj.p value p<0.05) in both activity and resting in comparison to control in red, and significantly expressed genes (adj. p<0.05) either during activity or resting time.

a


Control Activity

b


Control Resting

c

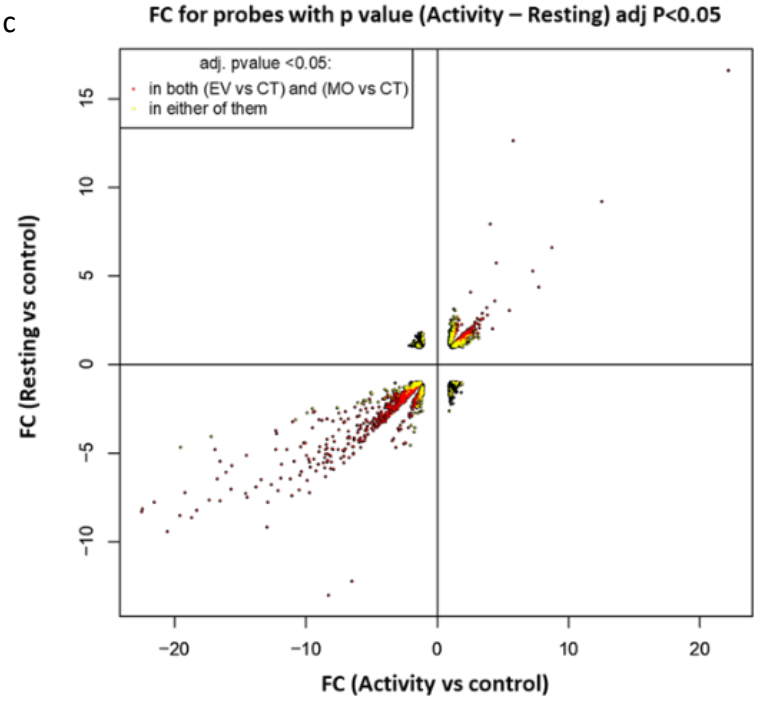
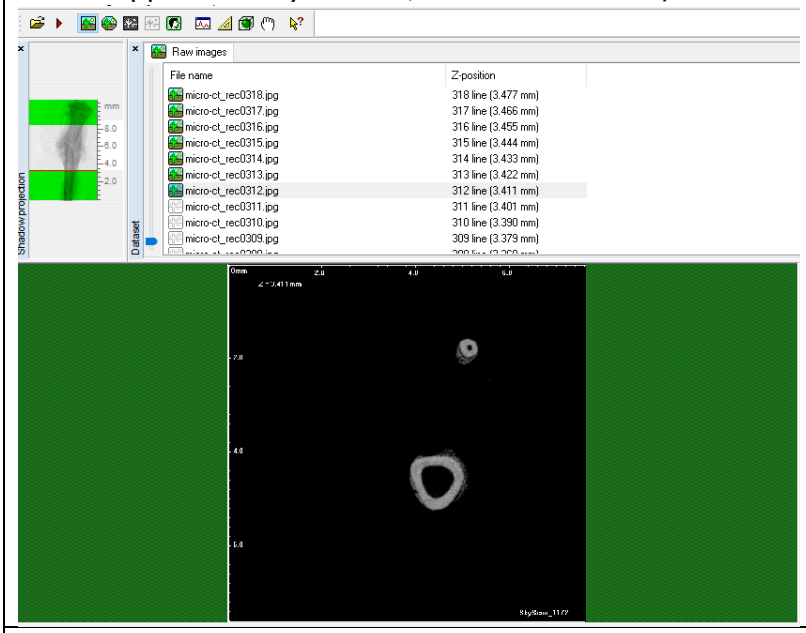
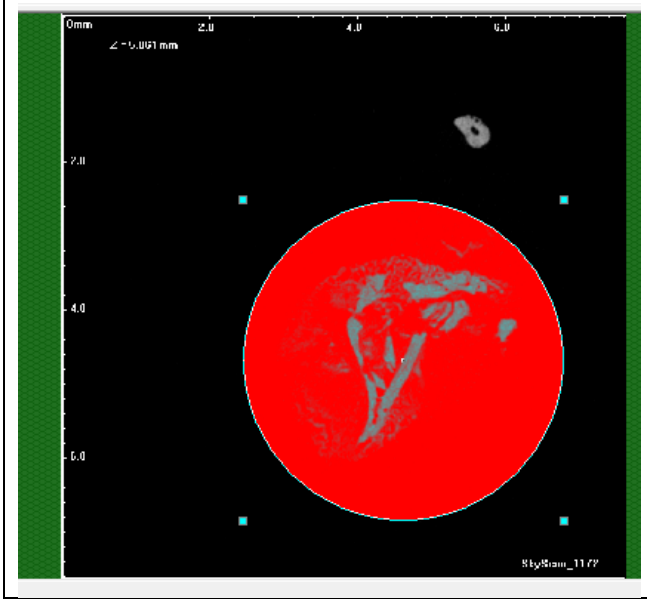

FC for probes with p value (Activity – Resting) adj P<0.05
adj. pvalue <0.05:
in both (EV vs CT) and (MO vs CT)
in either of them
FC (Resting vs control)
FC (Activity vs control)
15
10
5
0
−5
−10
−20
−10
0
10
20

Table 13-5S: µ CT 3D Reconstruction

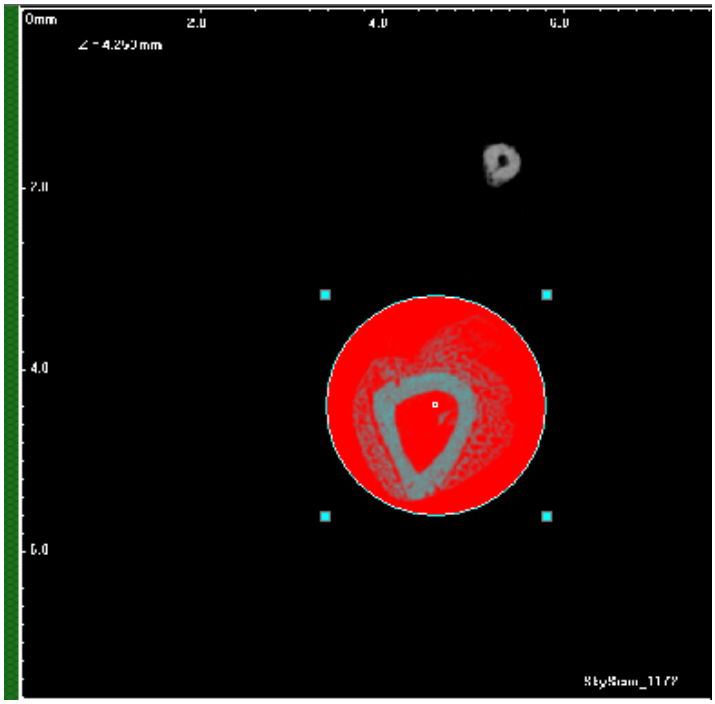
1- Open CTAn software(CT-Analyser; Bruker micro-CT, Kontich, Belgium).
2- Click folder button, load reconstructed CT data.
3- On Raw Image, Select the top and bottom section (from the fracture center, approximately ±2mm, end of bone callus).



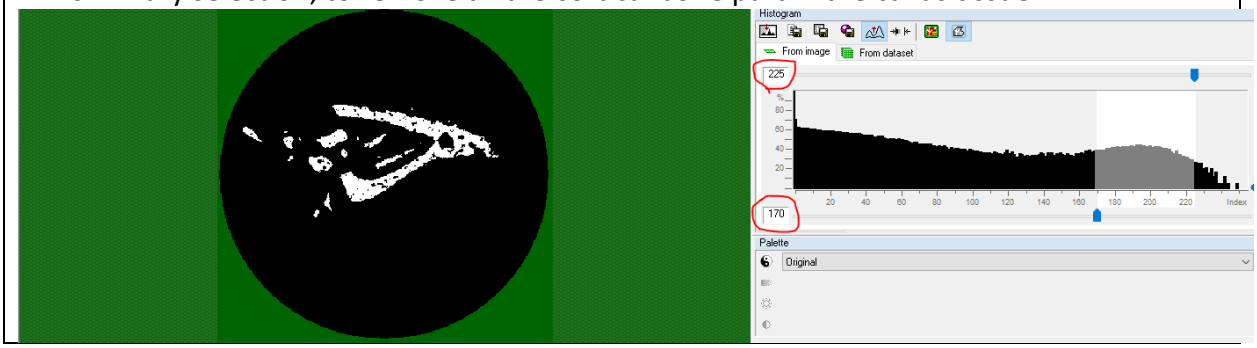
4- Select the biggest callus area, click on <Region of Interest (ROI, and choose all callus area



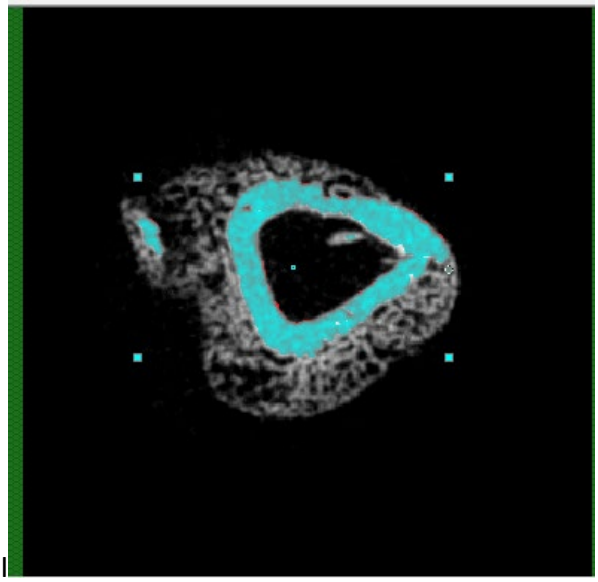
5- Check on other section, whether all the callus is included within ROI. Refine the ROI when move up or down to all the section.



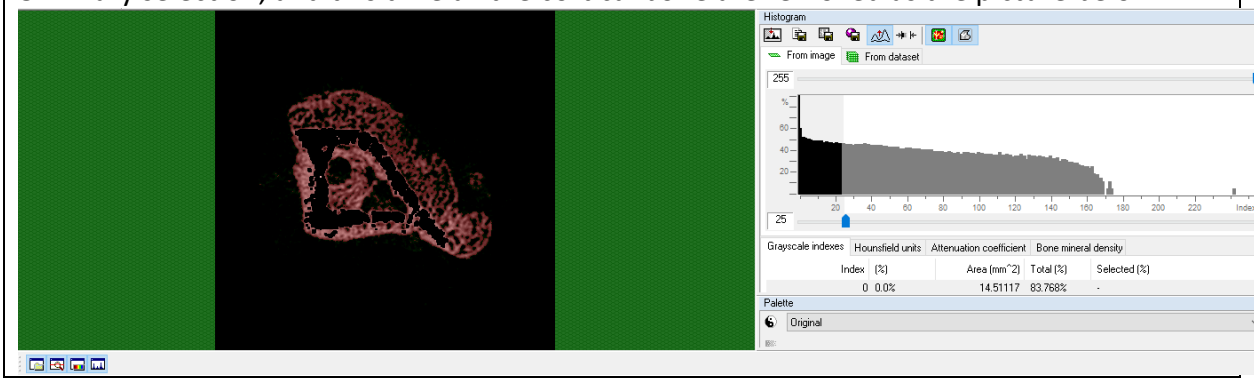
6- Binary selection; to remove all the cortical bone part in the callus tissue



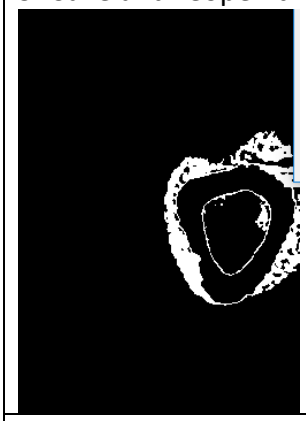
7- custom processing, then we will see the cortical bone ROI image is overlap with our current ROI



8- Binary selection, and this time all the cortical bone are removed as the picture below.



9- Save and reopen the binary data.



10- Custom processing, open the internal tab and choose 3D analysis for BV/TV, Trabecular bone number , thickness and spacing.

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14 Appendix III- Questionnaire and documents of clinical trial protocol

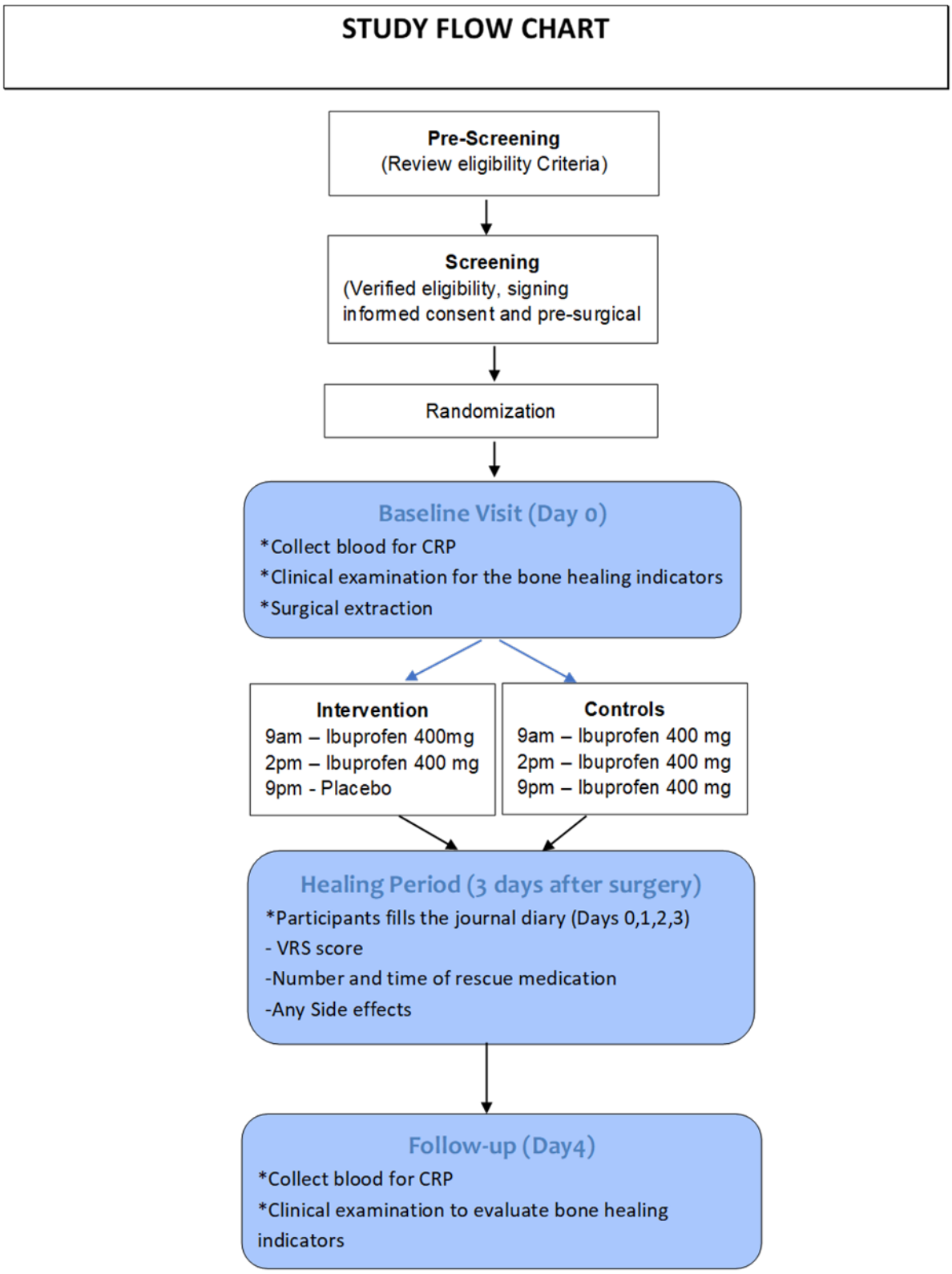

Figure 14-1S: Study flow chart

Table 14-1S:Schedule of enrolment, interventions and assessments(as per SPIRIT) (239)

	Enrolment	Allocation	Post allocation				
TIMEPOINT**	***-3 Day***	**Day0 (before surgery)**	***Day0 (after surgery)***	***Day 1***	***Day 2***	***Day 3***	***Follow up (Day 4)***
ENROLMENT:							
Eligibility screen	X						
Informed consent	X						
Allocation		X					
INTERVENTIONS:							
Group1-NSAID 3 times/day	←	—	—	—	→		
Group2-NSAID in the morning only	←	—	—	—	→		
ASSESSMENTS:							
VAS			X	X	X	X	
Type of food			X	X	X	X	
Number and time of rescue medication			X	X	X	X	
Side effect			X	X	X	X	
C-RP		X					X
MID		X					X
DHC		X					X
DHS		X					X

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Jordan University of Science and Technology

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Date: .....

الموافق: ٢٠١٧/٨/٢٩ م

الأستاذ الدكتورة عميد كلية طب الأسنان المحترمة
جامعة العلوم والتكنولوجيا الأردنية

تحية طيبة وبعد،،،

إشارةً الى البحث العلمي المُرسل إلكترونياً رقم (٢٠١٧/٣٩٣)، والمقدّم من الدكتور زيد زهير تميمي، بعنوان:

Efficacy of Non-Steroidal Anti-Inflammatory (Ibuprofen) Chronotherapy in Healing After Mandibular Third Molar Surgical Extraction? A Randomized Clinical Trial

يرجى إعلامكم بموافقة لجنة البحث على الانسان على إجراء البحث العلمي المُشار إليه أعلاه، على أن يتم التقيّد بالشروط التالية:

١. الإلتزام بسياسة البحث العلمي في المستشفى (رقم السياسة GM7601).
٢. الحفاظ على سرية المعلومات وأن لا تستخدم الا لغايات البحث العلمي.
٣. تزويدنا بنسخة من استمارة الإقرار بالموافقة للمشاركين في البحث، والاحتفاظ بنسخة أخرى مع الباحث لإبرازها عند الحاجة، مع الإلتزام بنموذج الإقرار بالموافقة على المشاركة في البحث المُعتمد من قبل اللجنة (مرفق نسخة مع القرار).
٤. تُعتبر الموافقة ملغاة تلقائياً بعد مرور عام من الحصول على موافقة لجنة أخلاقيات البحث على الإنسان (IRB)، أوفي حال عدم تزويد اللجنة بنتائج البحث. (مرفق مع القرار نموذج إغلاق البحث)

وتفضلوا بقبول فائق الاحترام،،،

رئيس لجنة البحث على الانسان

الأستاذ الدكتور يوسف القاعود

م.ر/ منسق لجنة البحث على الإنسان

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عنوان البحث: .....

.....

- قبل موافقتي على البحث تم اعلامي من قبل الباحث بما يلي :
  ١- موافقة الجهات المعنية في المستشفى على اجراء البحث.
  ٢- اهداف البحث واجراءاته.
  ٣- اية اخطار ممكنة ومتوقعة وأية مضايقات او منافع ناتجة عن البحث.
  ٤- اية اجراءات او علاجات بديلة ومحتملة.
  ٥- احتمال خطورة غير متوقعة.
  ٦- اية تعويضات او علاج طبي مؤمن لي في حال حدوث اذى او ضرر ما نتيجة البحث.
  ٧- المدة الزمنية اللازمة للبحث .
  ٨- كيفية الحفاظ على سرية المعلومات.
  ٩- الحالات التي ممكن ان تحث الباحث على ايقافي عن المشاركة في البحث.
  ١٠- اي جهد اضافي ممكن ان ابذله لغاية البحث.
  ١١- ماذا يحصل في حال قررت ان اتوقف عن المشاركة في البحث.
  ١٢- متى يتوجب اعلامي باستنتاجات جديدة ممكن ان تؤثر على عزيمتي في المشاركة في البحث.

- إذا كان لديك اسئلة ما تتعلق بحقوقك كمشارك في هذا البحث او فيما يتوجب عليك عمله في حال اصابتك بأذى او بضرر فمن الممكن الاتصال في اي وقت من الاوقات بـ
  ..... على الرقم .....

- مشاركتك في هذا البحث اختيارية فلن تعاقب أو تخسر اية منافع في حال قررت عدم المشاركة او التوقف عن المشاركة في اي وقت .
- بمجرد توقيعك على هذا المستند فإنت تقر بأنك توافق اختيارياً على المشاركة في هذا البحث وان المعلومات المدونة اعلاه قد شرحت لك بالكامل.

التاريخ	اسم المشارك	توقيعه .....
التاريخ	اسم الشاهد الاول	توقيعه .....
التاريخ	اسم الشاهد الثاني	توقيعه .....
التاريخ	اسم المشرف على البحث	توقيعه .....

- ملاحظة هامة: يجب حفظ نسخة من هذا النموذج في الملف الطبي للمشارك

Subject Information **and Informed Consent Form**

Protocol Code:	**ICF Version:**	**ICF Code:**
Subject Code:		

Informed Consent for Participation in a Clinical study

Please carefully read the following information about nature, significance, procedure and consequences of the planned study before you decide to take part. Please feel free to ask us if there is anything that is not clear or if you would like more information. We are here to answer any questions that you have at any time during the study.

- **The aim of this trial is to** compare the effect of chronotherapy (Time-specified dose) of Ibuprofen on the healing after third molars extraction
- The conduct of clinical studies in the development of a drug for use in humans is inevitable. This study is conducted to clinically verify that the potential product is effective at the morning dose than evening for the treatment of inflammation conditions after third molar extraction.
- The study will be conducted in accordance with the internationally established guidelines for good clinical practice (GCP) and the Declaration of Helsinki. The study has been submitted to an Independent Ethics Committee, who has returned a positive vote/favorable opinion. The responsibility for the study remains with the principal investigator, **dr Zaid Tamimi**
- During a clinical study, a participant's personal data and medical findings are collected. These data are forwarded, stored and reviewed according to legal Jordanian regulations. Prior to participation in the clinical study this requires your unsolicited consent.

- **With my signature, I confirm that I have had sufficient time to read and understand the information provided in this Subject Information. All my questions were answered to my satisfaction**
- **I agree that data/medical data collected within the scope of this study are** stored on paper **and on electronic data carriers and are forwarded in an anonymous form** to the sponsor of **the study for clinical evaluation and the responsible regulatory authority** Jordan Food and **Drug Administration for review of the correct performance of the study.**

I also agree to the review of my data collected by the investigator by persons authorized and sworn to secrecy by the sponsor, the responsible national (and foreign) regulatory authority or the responsible federal authority as far as this is necessary for the survey of the study. For this procedure/measure I release my investigator of his obligation to medical confidentiality as in the above **mentioned conditions.**

☐ **I agree** ☐ **I do not** agree

Participant Name ---------- Birth date: _____

National No. (Jordanian)/Passport No. (Other nationality): _____

Address: ❑ Home ----------

❑ **Work** ----------

Telephone No.: ❑ **Land line** ----------

❑ **Mobile** ----------

Participant Sign. ---------- Date: _____.

Name: _____

Address: _____

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You may potentially receive no benefit from this study, but it will have beneficial returns to society. Th will compare the effect of chronotherapy (Time-specified dose) of Ibuprofen on the healing after third extraction to relief inflammation to provide later it to the market to be used by patients

A) Sponsor's address:

Jordan University of Science
and Technology represented by
doctor Zaid Tamimi

Study Center:

The clinical part of this study will be performed at King Abdullah University Hospital and Dental Clinic Teaching Center at Jordan University of Science and Technology, Irbid, Jordan. Tel: + +962 2 7200600

B) Trial Substance:

Serodase is a drug that has been approved by the Jordanian Food and Drug Administration (Ministry of Health) since the year 1999.

- Serratiopeptidase is the active ingredient which has been in the market since the year 1960
- Administered dose 1-2 soft gel (400 mg each tablet) four times daily

- **Side effects:**

All medications may cause certain side effects and discomforts. Not all NSAIDs have the same safety profile; Ibuprofen has the most favorable GI safety profile of all NSAIDs, The study doctor will be directed you to take this medication, remember that he has judged that the benefit to you is greater than the risk of side effects. Most people using this medication do not have serious side effects. Ibuprofen at normal doses usually has no side effects. Most people using this medication do not have serious side effects.
Ibuprofen rarely cause side effect such as unexplained stomach pain (abdominal pain) or other abnormal stomach symptoms, indigestion, heartburn, feeling sick and/or vomiting. Allergic reactions (e.g. Unexplained wheezing, shortness of breath, skin rash, itching or bruising,) Severe sore throat with high fever, Yellowing of the eyes and/or skin, Blurred or disturbed vision , Fluid retention , Very rarely Ibuprofen tablets may cause aseptic meningitis, severe skin reactions (e.g., Stevens-Johnson syndrome), small increased risk of heart attack (myocardial infarction) or stroke , Ibuprofen tablets has also been shown to sometimes worsen the symptoms of Crohn's disease or colitis

The investigator will inform you about any new information related to the investigational product.

Please immediately inform your investigator of any side effects or impairments to your health that you may experience.

Subject Information and Informed Consent Form

نموذج معلوملت الدراسة و اقرار المشاركة

Protocol Code:	ICF Version:	ICF Code:
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Conditions of entry:

There are some general considerations that should be emphasized on before signing the informed consent form and before testing your suitability to participate in this trial.

I am here by the under signed confirm and agree on the following:

- To the best of' my knowledge. My health status is good. l do not need any treatment and all the information that I provide about my medical history is accurate.
- I am between 18-35 years old.
- I have not donated participated in any other clinical study within 8 weeks before enrolling in this study.
- I accept to undergo a dental physical checkup and examination by the study dentist.
- I do understand that according to the results of these investigations I will be included or excluded from the study.
- I agree to commit to the instructions given to me by the principal investigator and/or the co-investigators.
- **I have received a copy of the general instructions that explains study activities and the obligations I *have* during the study.**

Trial Procedures:

66 participants are taking part in this study for **5 days**

Confidentiality:

By taking part in this study, you consent that only the information that is necessary for the analysis and evaluation of the study will be collected by **The First for Research and Development L.L.C Company.** and its authorized representative. The information will not identify you by name but by a number and your initials. If the result of the study published, your identity will remain confidential.

Your anonymous medical information will be kept by the study investigators and **The First for research and development L.L.C Company** Your Information will be transferred into a computer data base and processed to allow the result of this study to be analyzed and reported or published. You have the right to access, through your study doctor, all the information collected about you and If applicable, ask for corrections, however in order to protect the scientific integrity of the study,

Phone: _____

Gender: 1☐ Female 2☐ Male **Data of birthday:** ___ ___ / ___ ___ / ___ ___ ___ ___
DD MM

Protocol Code:	ICF Version:	ICF Code:
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the treatment you received in this study needs to remain unknown (blinded) until the study data is analyzed. Only authorized representatives of **The First for Research and Development L.L.C "Company.** The institutional Review Board that approved this study and the regulatory Authorities (Jordan Food and Drug administration/Ministry of Health) will have direct access to your medical records. This; is, Necessary to check that the study is being performed correctly and that the information collected about you is accurate. All personnel accessing your records are required to respect your Confidentiality at all times.

If you should withdraw from the study, study data collected prior to your withdrawal may still be processed along with other data collected as part of the clinical study. However, you have the right to require that no new information is collected for the study data.

Authorizing the use of medical information

The Clinical study may only be performed by collecting and using, your medical information. National and international data protection regulations give you the right to control the use of your medical information. Therefore by signing this consent form you specifically authorize your medical information to be checked, transferred and processed as follows: The authorized representatives of **The First for Research and Development L.L.C "Company**. The institutional Review Board, and the regulatory Authorities' inspectors may review your medical information by direct access to your medical records. Study data. Including your anonymous medical information may be processed. Which means it will be collected, entered into computer databases, verified, analyzed, printed and reported as Necessary for legitimate scientific purposes, including use in future medical or pharmaceutical Research.

Risk and safety procedure:

- Please note that every medicinal drug can in, exceptional cases, cause side effects which are unexpected or which have not previously been observed.
- For your safety ,side effects are examined regularly during the complete study

Withdrawal of consent or premature termination:

Every participants has the right to withdraw his consent to participant in this study at any time without having to give a reason and without suffering any consequences. For your safety and in your own interest however, it is important for you to undergo the final examination if you took the drug dose.

The investigator can stop participation at any time without your consent if it appears to be medically harmful to you if he/she considers the outcome of the trial at risk due to your failure to follow directions correctly. The study may also be terminated by the sponsor and/or the Institutional Review Board/Independent Ethics Committee.

Insurance:

- The sponsor of the study (**Jordan University of Science and Technology)** represented by the first for research and Development Company will buy insurance to cover study related injuries for you if you are taking part in this study.

Subject Information and Informed Consent Form

نموذج معلوملت الدراسة و اقرار المشاركة

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☐The insurance company will pay compensation where the injury probably resulted from:

- A drug being tested or administered as part of the trial protocol.
- Any test of procedure you received as part of the trial>

The insurance Company will not be bound by these guidelines to pay compensation where:

- The injury resulted from a drug or procedure outside of the trial protocol.
- The protocol was not followed.
- There was contributory negligence by the patient

Contacts for Information

- If you have any questions about the study, please contact clinical site responsible person Dr.Zaid Tamimi.
- If you have an injury/side effect caused by the study, please contact the **Jordan University of Science and Technology represented by doctor Zaid Tamimi**079503670

Expenses and payments

You will not receive payments for taking part in this study, but the study drug will be made available to you at no charge, the extraction procedure will be free of charge as well, and you will not be required to pay for any study procedures or visits.

Consent Form

• I herewith give me my consent to participant in this trial voluntarily, based on the information about the trial provided to me by the investigator and all my questions were answered to my satisfaction. • I have received a copy of the subject information. I have the right to obtain a copy of this informed consent form.

Participant name & Signature:	Date:

Subject Information and Informed Consent Form

نموذج معلومات الدراسة و اقرار المشاركة

Protocol Code:	ICF Version:	ICF Code:
Subject Code:		

.st witness name& Signature:	Date:
.nd witness name& Signature:	Date:
Clinical Investigator name& Signature:	Date:
Principle investigator name& Signature:	Date:

Subject Information and Informed Consent Form

نموذج معلومات الدراسة و اقرار المشاركة

Protocol Code:	ICF Version:	ICF Code:
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اقرار الموافقة على المشاركة في دراسة سريرية

نرجو منك أن تقضي وقتا كافيا لمطالعة المعلومات التالية بتمعن قبل أن تقرر المشاركة وهي معلومات تتعلق بطبيعة وأهمية ., ومجريات ونتائج الدراسة المنوي إجراؤها. ولك الحق و الحرية في توجيه آية أسئلة أو االستفسار عن أية معلومة غير واضحة في أي وقت أثناء الدراسة. و من واجبنا االجابة عن كل تساؤ الاتك

- إن الهدف من هذه الدراسة هو التحقق من فاعلية المستحضر ايبوبروفين (جرعه محدد من الوقت)في عالج االلتهابات غير البكتيريه الناتجه بعد الخلع الجراحي للضرس العقل

لا بد من اإلشارة إلـى أن اللجوء إلى الدراسات السريرية فـي سياق تطوير مستحضر دوائي لصالح صحة الانسان هو أمر الا بد منه . وتستهدف هذه الدراسة الحصول على إثبات سريري لفاعلية مستحضر سيروداز

- سيتم تنفيذ الدراسة طبقا لألنظمـة الدولية المعتمدة فـي الممارسة السريرية الصحيحة وميثاق هلسنكي . وقد تم عرض الدراسة على لجنـة أخالقيات مستقلة . وقامت هذه اللجنة بإبداء رأي إيجابي بشأنها والمصادقة على اجر انها , وقد أنيطت مسؤولية الدراسة بالباحث (لمسؤول) , **الدكتور زيد التميمي**
سوف يلم الحصول في سباق الدراسة السريرية على بيانات شخصية خاصة لك و على نتائج طبيه. كما سوف يتم تقديم و عرض وحفظ , ومعاينة هذه البيانات وفق مقتضيات القوانين والتعليمـات االردنيـه النافذة . الامر الذي يتطلب موافقتكم الطوعية على ذلك قبل المشاركة بالدراسة السريرية
- ان توقيعي لهذا النموذج يثبت أنه كان لدي الوقت الكافي لقراءة ة استيعاب المعلومات الموجهة الى المشاركين بهذا السياق، و أنني حصلت على أجوبة شفافة لكل تساؤلاتي.
- أوافق على حفظ البيانات/البيانات الطبية التي يتم الحصول عليها في سياق الدراسة السريرية في وثائق كتابية و باستخدام الوسائط الالكترونية، كما أوافق على تقديمها، وبدون تعيين هويتي الشخصية الى راعي الدراسة لغايات التقييم السريري، و المؤسسة الاردنية العامة للغذاء والدواء للنظر في صحة التنفيذ الدراسة.
- كما أوافق على معاينة البيانات الخاصة بي التي يقوم الباحث (مسؤول الدراسة) بالحصول عليها من قبل أشخاص يفوضهم الباحث تحت اتفاقية الحفاظ على سرية المعلومات: و مراجعتها من قبل السلطات الوطنية والاجنبية ذات العالقة . وذلك بالقدر الضروري لتقييم الدراسة . ونظرا لهذا الاجراء/التدبير فإنني أعفي الجهة المسؤولة عن الدراسة التي أشارك فيها من مسؤولية الالتزام بالسرية الطبية وحسب الظروف اعلاه.

موافق ☐ غير موافق ☐

اسم مشترك الرباعي Name:---------- تاريخ الميلاد:----------

الرقم الوطني(للاردنيين)/او رقم جواز السفر(لغير الاردنيين):----------

عنوان: - المنزل ----------

- العمل ----------

رقم الهاتف: - الارضي----------

- الخلوي ----------

توقيع المشارك:---------- التاريخ:----------

Subject Information and Informed Consent Form

نموذج معلومات الدراسة و اقرار المشاركة

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و اذا كان ذلك منطبقا و طلب تصحيحها اذا انطبق ذلك و لكن من اجل حماية سلامة المحتوى العلمي للدراسة فان العالج الذي تلقيته هذه الدراسة يجب ان يظل غير معروف (معمى) حتى يتم تحليل بيانات الدراسة.

فقط الممثلين المفوضين من الشركة الاولى للبحث و التطوير الصيدلاني, اللجنة المؤسسية لمراجعة البحوث التي وافقت على هذه الدراسة والسلطات المحلية (المؤسسة العامة للغذاء والدواء / وزارة الصحة) سيكون لهم حقت الوصول المباشر إلى السجالت الطبية الخاصة بك. وهذا أمر ضروري للتأكد من أنه سيتم تنفيذ هذه الدراسة بشكل صحيح. وأن المعلومات التي تم جمعها عنك دقيقة. ويعين على جميع الموظفين المخولين بالوصول إلى السجلات الخاصة بك احترام سرية معلوماتك ب جميع الأوقات.

إذا كنت تريد أن تنسحب من هذه الدراسة. فالبيانات التي تم جمعها قبل انسحابك لا يزال من الممكن معالجتها مع غيرها من البيانات التي تم جمعها كجزء من الدراسة السريرية. ومع ذلك. لديك الحق في أن تشترط ان لا يتم جمع أية معلومات جديدة لبيانات الدراسة.

الموافقة على استخدام المعلومات الطبية

هذه الدراسة السريرية يمكن إجراءها فقط عن طريق جمع واستخدام المعلومات الطبية الخاصة بك , والسريرية. القوانين الوطنية والدولية لحماية البيانات تعطيك الحق في مراقبة استخدام المعلومات الطبية الخاصة بك. ولذلك , من خلال توقيع نموذج الموافقة هذا فانك تعطي الاذن بفحص و نقل و معالجة معلوماتك الطبية على النحو التالي:

- الممثلين المفوضين من الشركة الاولى للبحث و التطوير الصيدلاني, واللجنة المؤسسية لمراجعة البحوث, ومفتشي السلطات التنظيمية يمكنهم مراجعة المعلومات الطبية الخاصة بك عن طريق الوصول المباشر إلى سجلاتك الطبية.
- قد يتم معالجة بيانات الدراسة , بما في ذلك المعلومات الطبية وأية عينات طبية مأخوذة دون الافصاح عن هويتك , مما يعني سيتم جمعها وادخالها في قواعد البيانات الحاسوبية للتحقق منها وتحليلها وطباعتها والتصريح عنها وفقا للأغراض العلمية المشروعة. بما في ذلك الاستخدام المستقبلي في البحوث الطبية أو الصيدلانية.

المخاطر و اجراءات السالمة:

- يرجى االخذ بعين الاعتبار أن كل مستحضر دوائي قد يسبب , في أحوال استثنائية . اثار جانبية لا يمكن توقعها لم يسبق مالحظتها من قبل.
- ومن أجل الحفاظ على سالمتك سيتم فحص التأثيرات الجانبية للعقار بانتظام أثناء سير الدراسة

الانسحاب أو و قف مشاركتك من الدر اسة:

يحق لك التراجع عن موافقتك المشاركة في هذه الدراسة و الانسحاب من الدراسة في أي وقت بدون إبداء سبب لذلك وبدون التعرض لأية عواقب . إال أنه من الأهمية بمكان , حفاظا على سلامتك ومصلحتك الشخصية ،الخضوع للفحص النهائي في حال أخذ جرعة الدواء.

وبمعزل عن ذلك , فان للباحث (مسؤول الدراسة) الحرية في وقف مشاركتك في أي وقت إذا كان من شأنها الاضرار بصحتك , أو إذا بدا له/لها أن هناك خطرا يهدد حصيلة الدراسة بسبب تقاعسك عن اتباع التعليمات بدقة . ويمكن إنهاء الدراسة أيضا من قبل راعي الدراسة و/أو لجنة المراجعة المؤسسية/لجنة الاخالقيات المستقلة.

التامين:

- راعي الدراسة (الجامعه الاردنيه للعلوم و التكنولوجيا) سوف تعمل على شراء التامين لتغطية الاصابات ذات الصلة بالدراسة اذا كنت ستشترك في هذه الدراسة و شركة التامين سوف تدفع التعويضات اذا كانت الاصابة ناتجة عن :

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إذا كنت تريد أن تنسحب من هذه الدراسة. فالبيانات التي تم جمعها قبل انسحابك لا يزال من الممكن معالجتها مع غيرها من البيانات التي تم جمعها كجزء من الدراسة السريرية. ومع ذلك. لديك الحق في أن تشترط ان لا يتم جمع أية معلومات جديدة لبيانات الدراسة.

الموافقة على استخدام المعلومات الطبية

هذه الدراسة السريرية يمكن إجراءها فقط عن طريق جمع واستخدام المعلومات الطبية الخاصة بك , والسريرية. القوانين الوطنية والدولية لحماية البيانات تعطيك الحق في مراقبة استخدام المعلومات الطبية الخاصة بك. ولذلك , من خلال توقيع نموذج الموافقة هذا فانك تعطي الاذن بفحص و نقل و معالجة معلوماتك الطبية على النحو التالي:

- الممثلين المفوضين من الشركة الاولى للبحث و التطوير الصيدلاني, واللجنة المؤسسية لمراجعة لمراجعة البحوث, ومفتشي السلطات التنظيمية يمكنهم مراجعة المعلومات الطبية الخاصة بك عن طريق الوصول المباشر إلى سجلاتك الطبية.
- قد يتم معالجة بيانات الدراسة , بما في ذلك المعلومات الطبية وأية عينات طبية مأخوذة دون الافصاح عن هويتك , مما يعني سيتم جمعها وادخالها في قواعد البيانات الحاسوبية للتحقق منها وتحليلها وطباعتها والتصريح عنها وفقا للأغراض العلمية المشروعة. بما في ذلك الاستخدام المستقبلي في البحوث الطبية أو الصيدلانية.

المخاطر و اجراءات السالمة:

- يرجى االخذ بعين الاعتبار أن كل مستحضر دوائي قد يسبب , في أحوال استثنائية . اثار جانبية لا يمكن توقعها لم يسبق مالحظتها من قبل.
- ومن أجل الحفاظ على سالمتك سيتم فحص التأثيرات الجانبية للعقار بانتظام أثناء سير الدراسة

الانسحاب أو و قف مشاركتك من الدر اسة:

يحق لك التراجع عن موافقتك المشاركة في هذه الدراسة و الانسحاب من الدراسة في أي وقت بدون إبداء سبب لذلك وبدون التعرض لأية عواقب . إال أنه من الأهمية بمكان , حفاظا على سلامتك ومصلحتك الشخصية ،الخضوع للفحص النهائي في حال أخذ جرعة الدواء.
وبمعزل عن ذلك , فان للباحث (مسؤول الدراسة) الحرية في وقف مشاركتك في أي وقت إذا كان من شأنها الاضرار بصحتك , أو إذا بدا له/لها أن هناك خطرا يهدد حصيلة الدراسة بسبب تقاعسك عن اتباع التعليمات بدقة . ويمكن إنهاء الدراسة أيضا من قبل راعي الدراسة و/أو لجنة المراجعة المؤسسية/لجنة الاخالقيات المستقلة.

التامين:

- راعي الدراسة (الجامعه الاردنيه للعلوم و التكنولوجيا) سوف تعمل على شراء التامين لتغطية الاصابات ذات الصلة بالدراسة اذا كنت ستشترك في هذه الدراسة و شركة التامين سوف تدفع التعويضات اذا كانت الاصابة ناتجة عن :

Subject Information and Informed Consent Form

نموذج معلوملت الدراسة و اقرار المشاركة

Protocol Code:	ICF Version:	ICF Code:
Subject Code:		

- الدواء الذي تم اختباره او الذي يعطى كجزء من بروتوكول الدراسة.
- أى فحص او اجراء تلقته كجزء من هذه الدراسة.
- و بناء على هذه التعليمات لن تلتزم شركة التامين بدفع التعويضات في الحاالت التالية:
- الاصابة الناتجة عن دواء او اجراء خارج بروتوكول الدراسة
- عدم اتباع البروتوكول
- كان هناك اهمال من قبل المريض.

الاستعلام عن الدراسة:

- إذا كانت لديك أي أسئلة عـن الدراســة. يرجى االتصـال بمسؤول القسـم السريري الدكتور زيـد التميمـي

079503670د خالد السالم رئيس لجنة االخالقيات (027200600)

- إذا حدثت لك أي إصابة ناتجة عن الدراسة, يرجى االتصال بجامعة العلوم و التكتنولوجيا ممثلة بالدكتور زيد التميمي 079503670

التكاليف و المدفوعات:

لن يدفع مقابل مشاركتك فـي هذه الدراسـة و لكن سيتم توفير ادوية الدر أسـة للك بدون مقابل و ستكون عملية خلع الاضراس مجانية و

Subject Information and Informed Consent Form

نموذج معلوملت الدراسة و اقرار المشاركة

Protocol Code:	ICF Version:	ICF Code:
Subject Code:		

نموذج الموافقة

• أوافق على المشاركة في هذه الدراسة طوعا و بمطلق اختياري و رغبتي, وذلك بعد الاطلاع على معلومات الدراسة و الحصول على أجوبة شافية لكل تساؤلاتي التي من قبل الباحث (مسؤول الدراسة). • وأصرح بأنني تسلمت نسخة من المعلومات الموجهة إلى المشاركين, ويحق لي عند رغبتي الحصول على نسخة من نموذج الموافقة على المشاركة في دراسة التكافؤ الحيوي.

اسم التوقيع المشارك:	**التاريخ:**

	التاريخ:
اسم و توقيع شاهد ثاني:	**التاريخ:**
اسم و توقيع القسم السريري:	**التاريخ:**
اسم و توقيع الطبيب المسؤول:	**التاريخ:**

Date of report:		Study No.:	Ser

Investigator Name:		Site No.:	0	Subject initials		Subject no.	

1.Site contact Information			
Coordinator name:			
Site address:	King Abdullah University Hospital and dental clinic teaching center at Jordan university of science and technology P.O.Box:360001		
Email address:	susnak@just.edu.jo		
Telephone No:	+962 2 72006000	Fax number:	+962 2 7095777
Data site became aware of pregnancy:			

2. Subject information				
Medication No. Batch No.	CRF ID	DOB	Start date of last menses Date pregnancy confirmed	Anticipated date of childbirth N/A ☐

3. Details of IMP(s)				
Study design	Treatment &daily dose	Route Formulation	Start date dd MMM yyyy	Stop date dd MMM yyyy
☐ Double Blind code not-broken	Treatment UNK Dose:			
☐ Double Blind code broken				
☐ Open Trial				
☐ No study drug	No study medication			

4.Pregnancy outcome			
☐ Not known at this time	☐ Included abortion	☐ Still birth	☐ Neonatal death
☐ Normal/healthy baby	☐ Spontaneous abortion	☐ Birth defects	

5.Treating physician
Name& address/ contact details incl. phone and fax:

6. Subject consented to pregnancy monitoring	
☐ Yes	☐ No

7. Ethics Committee notification	
IRB/IEC that granted the study approval? ☐ Yes, give a date	Has a copy of this report been sent to the ☐ No, planned submission date:

Name of reporter: _____

Informed Consent

Please note: written informed consent must be given before any specific procedures take place or any current therapy discontinued for the purposes of participation in this study.

Has the subject freely given written informed consent? $_1$☐ Yes $_2$☐ No

Screening

Has the subject body temperature been taken?	☐ Yes
Has the subject blood pressure been taken?	☐ Yes
Has the subject pulse been taken?	☐ Yes
Has the OPC been taken?	☐ Yes

1. Does the subject satisfy the inclusion and exclusion criteria?
$_1$☐ Yes $_2$☐No

2. Have all screening procedures been completed?
$_1$☐ Yes $_2$☐No

3. Is the subject willing to proceed? Is the subject able to continue?
$_1$☐ Yes $_2$☐No

4. Have the dosing instructions been explained to the patient?
$_1$☐ Yes $_2$☐No

If yes to the questions above, please complete the questionnaire in the next page and schedule the surgery.

Interviewer: Now I would like to ask some questions about your lifestyle

1. What is the highest level of education you completed?
No schooling..... $_1$☐
Primary school..... $_2$☐
Did not complete high school (grade 7 to 11)..... $_3$☐
Graduated from high school (grade 12)..... $_4$☐
Graduated from vocational or trade school..... $_5$☐
Graduated from college (2 year course)..... $_6$☐
Graduated from university..... $_7$☐
Don't know..... $_8$☐

2. What was your family annual income?

Less than 6000 JD ..... 1☐
6000JD - 12000JD ..... 2☐
12000 JD-18000 JD..... 3☐
18000 JD – 24000 JD ..... 4☐
24000 JD – 30000 JD..... 5☐
30000 JD - or more JD .....6☐

3. What is your occupation? Please describe what you do.

_____

4. Who do you live with?
I live by myself..... 1☐
I live in a dorm..... 2☐
I live with my family..... 3☐
I live with friends..... 4☐
Others ..... 5☐

5. Do / did you smoke cigarettes?
1☐ Yes 2☐ No (go to question…)

6. How many cigarettes do you smoke per day? _____

7. How long have you been smoking for? _____

8. Do / did you smoke shisha?
1☐ Yes 2☐ No (go to question…)

9. How long have you been smoking shisha for? _____

10. What is the overall levels of patients' oral hygiene? _____
Excellent (no visible plaque, inflammation, or calculus)..... 1☐
Very good (minimal plaque, without inflammation & calculus)..... 2☐
Good (minimal plaque, inflammation & calculus)..... 3☐
Poor (abundant plaque, minimal inflammation & calculus..... 4☐
Very poor (abundant plaque, minimal inflammation & calculus..... 5☐

3. Is the patient currently taken any medication? 1☐ Yes 2☐No

If yes, please describe all the medications that patients have been using:

_____
_____

Signed by: ---------- **Date:**

d	**d**	**m**	**m**	**m**	**y**	**y**	**y**	**Y**

Surgery day (VISIT 1)

Time_____ Date: ___ ___ / ___ ___ / ___ ___ ___ ___
DD MM YYYY

Has the subject body temperature been taken?	☐ Yes
Has the subject blood pressure been taken?	☐ Yes
Has the subject pulse been taken?	☐ Yes
Has the OPG been taken?	☐ Yes

1. What is the maximal interincisal distance which was measured using Vernier caliper_____mm

2. What are the DHC facial swelling measurements? _____

3. What are the DHS facial swelling measurements? _____

4. What are the DV facial swelling measurements? _____

5. What is the level of CRP? _____

6. What type of tooth impactions?

Vertical..... 1☐

Mesio-angular..... 2☐

Disto-angular ..... 3☐

Horizontal..... 4☐

Buccal lingual (any tooth that the crown overlaps the roots)..... 5☐

Others.....6☐

7. What time was the surgery performed? _____☐ No, s*urgery was cancelled*

8. How many anesthetic carpools were used? _____

9. Was there a lingual retraction?

1☐ yes 2☐ No, s**urgery was cancelled**

10. What was the duration of the surgery? _____minutes

11. What was the tooth number? _____

12. Any surgery complications? 1☐ yes 2☐ No

13. Was antibiotic prescribed? 1☐ yes 2☐ No

Follow-up visit (4 days after surgery, VISIT 2)

Time_____ Date: ___ ___ / ___ ___ / ___ ___ ___ ___
DD MM YYYY

11. What is the maximal interincisal distance which was measured using Vernier calipers? _____cm

12. What are the DHC facial swelling measurements? _____

13. What are the DHS facial swelling measurements? _____

14. What are the DV facial swelling measurements? _____

15. What is the level of CRP? _____

Please complete this form in case there is an accident

Medication	Total Daily Dose	Units	Reason	Start Date *(MM/DD/YYYY)*	Stop Date *(MM/DD/YYYY)*	Continuing
				___ ___/ ___ ___ / ___ ___ ___ ___	___ ___/ ___ ___ / ___ ___ ___ ___	☐
				___ ___/ ___ ___ / ___ ___ ___ ___	___ ___/ ___ ___ / ___ ___ ___ ___	☐
				___ ___/ ___ ___ / ___ ___ ___ ___	___ ___/ ___ ___ / ___ ___ ___ ___	☐
				___ ___/ ___ ___ / ___ ___ ___ ___	___ ___/ ___ ___ / ___ ___ ___ ___	☐
				___ ___/ ___ ___ / ___ ___ ___ ___	___ ___/ ___ ___ / ___ ___ ___ ___	☐
				___ ___/ ___ ___ / ___ ___ ___ ___	___ ___/ ___ ___ / ___ ___ ___ ___	☐
				___ ___/ ___ ___ / ___ ___ ___ ___	___ ___/ ___ ___ / ___ ___ ___ ___	☐
				___ ___/ ___ ___ / ___ ___ ___ ___	___ ___/ ___ ___ / ___ ___ ___ ___	☐
				___ ___/ ___ ___ / ___ ___ ___ ___	___ ___/ ___ ___ / ___ ___ ___ ___	☐
				___ ___/ ___ ___ / ___ ___ ___ ___	___ ___/ ___ ___ / ___ ___ ___ ___	☐
				___ ___/ ___ ___ / ___ ___ ___ ___	___ ___/ ___ ___ / ___ ___ ___ ___	☐
				___ ___/ ___ ___ / ___ ___ ___ ___	___ ___/ ___ ___ / ___ ___ ___ ___	☐
				___ ___/ ___ ___ / ___ ___ ___ ___	___ ___/ ___ ___ / ___ ___ ___ ___	☐

Has the patient experienced any Adverse Events since signing the Informed Consent? 1☐ Yes, specify below 2☐ No										
AE no	**Adverse Event** (diagnosis (if known) or signs/symptoms	**Start Date** dd/mm/yyyy **Time** (24 hour clock)	**Stop Date** dd/mm/yyyy **Time** (24 hour clock)	**Outcome** 1=Recovered 2=Recovered with sequelae 3=Continuing 4=Patient Died 5=Change in AE 6=unknown	**Severity** 1=Mild 2=Moderate 3=Severe	**Plausible relationship to Study Drug**	**Action taken with Study Drug** 1=None 2=Dose Reduction Temporarily 3=Dose Reduced 4=Discontinued Temporarily 5=Discontinued	**Withdrawn due to AE?**	**Serious AE (SAE)?**	**If SAE does it require immediate reporting? (See Protocol)?**
		☐☐ DD ☐☐ MM ☐☐☐☐ Year	☐☐ DD ☐☐ MM ☐☐☐☐ Year			1☐ yes 2☐ No		1☐ yes 2☐ No	1☐ yes 2☐ No	1☐ yes 2☐ No

OFF STUDY FORM

Date Off Study: ___ ___ / ___ ___ / ___ ___ ___ ___
(MM/DD/YYYY)

Date Last Study Medication Taken: ___ ___ / ___ ___ / ___ ___ ___ ___
(MM/DD/YYYY)

Reason Off Study (Please mark only the primary reason. Reasons **other than Completed Study** require explanation next to the response)

- ☐ Completed study
- ☐ AE/SAE **(complete AE CRF & SAE form, if applicable)**
  _____
- ☐ Lost to follow-up
  _____
  _____
- ☐ Non-compliant participant
  _____
  _____
- ☐ Concomitant medication
  _____
  _____
- ☐ Medical contraindication
  _____
  _____
- ☐ Withdraw consent
  _____
  _____
- ☐ Death **(complete SAE form)**
  _____
- ☐ Other
  _____
  _____

Journal Diary for *first day-surgery and the three days follow-up*

8:00 am **Remember to take the medication**

Pain Score (mark the pain intensity now)- refer to pain description guide

NO PAIN 0☐ 1☐ 2☐ 3☐ 4☐ 5☐ 6☐ 7☐ 8☐ 9☐ 10☐ WORST PAIN

2:00 pm **Remember to take the medication**

Pain Score (mark the pain intensity now)- refer to pain description guide

NO PAIN 0☐ 1☐ 2☐ 3☐ 4☐ 5☐ 6☐ 7☐ 8☐ 9☐ 10☐ WORST PAIN

9:00 pm **Remember to take the medication**

Pain Score (mark the pain intensity now)- refer to pain description guide

NO PAIN 0☐ 1☐ 2☐ 3☐ 4☐ 5☐ 6☐ 7☐ 8☐ 9☐ 10☐ WORST PAIN

Pain description guide	
Zero to one: no pain	Five to seven: pain interfere with concentration
One to three: pain can be ignored	Seven to nine: pain interfere with basic needs
Three to five: pain interfere with task	Nine to ten: pain required bed rest

Mark type of Food you have during the day?

Liquid **Semi msolid** **Solid**

Note: If you are taking (Panadol), please fill in the table

Number (tablets)	**Time**

Any complication or discomfort happened?

□YES □NO

8:00 am **Remember to take the medication**

Pain Score (mark the pain intensity now)- refer to pain description guide

NO PAIN	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐	9☐	10☐	WORST PAIN

2:00 pm **Remember to take the medication**

Pain Score (mark the pain intensity now)- refer to pain description guide

NO PAIN	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐	9☐	10☐	WORST PAIN

9:00 pm **Remember to take the medication**

Pain Score (mark the pain intensity now)- refer to pain description guide

NO PAIN	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐	9☐	10☐	WORST PAIN

Pain description guide	
Zero to one: no pain	Five to seven: pain interfere with concentration
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Three to five: pain interfere with task	Nine to ten: pain required bed rest

---

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Liquid **Semi msolid** **Solid**

Note: If you are taking (Panadol), please fill in the table

Number (tablets)	**Time**

Any complication or discomfort happened?

□YES □NO

8:00 am **Remember to take the medication**

Pain Score (mark the pain intensity now)- refer to pain description guide

NO PAIN	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐	9☐	10☐	WORST PAIN

2:00 pm **Remember to take the medication**

Pain Score (mark the pain intensity now)- refer to pain description guide

NO PAIN	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐	9☐	10☐	WORST PAIN

9:00 pm **Remember to take the medication**

Pain Score (mark the pain intensity now)- refer to pain description guide

NO PAIN	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐	9☐	10☐	WORST PAIN

Pain description guide	
Zero to one: no pain	Five to seven: pain interfere with concentration
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Three to five: pain interfere with task	Nine to ten: pain required bed rest

---

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Note: If you are taking (Panadol), please fill in the table

Number (tablets)	**Time**

Any complication or discomfort happened?

□YES □NO

8:00 am **<u>Remember to take the medication</u>**

Pain Score (mark the pain intensity now)- refer to pain description guide

NO PAIN 0☐ 1☐ 2☐ 3☐ 4☐ 5☐ 6☐ 7☐ 8☐ 9☐ 10☐ WORST PAIN

2:00 pm **<u>Remember to take the medication</u>**

Pain Score (mark the pain intensity now)- refer to pain description guide

NO PAIN 0☐ 1☐ 2☐ 3☐ 4☐ 5☐ 6☐ 7☐ 8☐ 9☐ 10☐ WORST PAIN

9:00 pm **<u>Remember to take the medication</u>**

Pain Score (mark the pain intensity now)- refer to pain description guide

NO PAIN 0☐ 1☐ 2☐ 3☐ 4☐ 5☐ 6☐ 7☐ 8☐ 9☐ 10☐ WORST PAIN

Pain description guide	
<u>Zero to one</u>: no pain	<u>Five to seven</u>: pain interfere with concentration
<u>One to three</u>: pain can be ignored	<u>Seven to nine</u>: pain interfere with basic needs
<u>Three to five</u>: pain interfere with task	<u>Nine to ten:</u> pain required bed rest

---

Mark type of Food you have during the day?

Liquid **Semi msolid** **Solid**

Note: If you are taking (Panadol), please fill in the table

Number (tablets)	**Time**

Any complication or discomfort happened?

□YES □NO