RELATIONSHIP OF TIMING OF DICLOFENAC AND CELECOXIB USE AND FRACTURE CALLUS MORPHOLOGY AND MORPHOMETRY IN A RAT MODEL

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DECLARATION

I hereby confirm that this thesis is my original work and has not been presented elsewhere for examination:

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DEDICATION

To my parents Leah Catherine Wangechi and Stephen Kigera; for always believing in me

To the love of my life Serah; for helping sculpt the softer side of me

To the apples of my eye Lemuel and Zemira; for teaching me patience and how to laugh again

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LIST OF ABBREVIATIONS

ALP	Alkaline Phosphatase
BMP	Bone Morphogenetic Proteins
COX	Cyclooxygenase
EDTA	Ethylenediaminetetraacetic acid
H&E	Haematoxylin and Eosin
IHC	Immunohistochemistry
IP	Intraperitoneal
NSAIDs	Non-steroidal anti-inflammatory drugs
MSCs	Mesenchymal Stem Cells
OTC	Over-The-Counter
H&E	Haematoxylin and Eosin (H&E)
MT	Masson's Trichrome (MT)
LR-PRP	Leucocyte-Rich Platelet-Rich Plasma
RUNX2	Runt-Related Transcription Factor 2
RANKL	Receptor Activator of Nuclear Factor-kB Ligand
TGF-β3	Transforming Growth Factor Beta 3
FGF-1	Fibroblast Growth Factor 1
SHH	Sonic Hedgehog
WNT	Wingless/Integrated

OPERATIONAL TERMS

Matrix – Extracellular Matrix

Cell Density – Numerical Cell Density as measured by Stereological techniques

SUMMARY

Background: Fractures are increasingly common worldwide partly due to road traffic collisions, workplace mechanization, and aging. The healing of fractures goes through a series of overlapping stages starting with the inflammatory phase. The use of non-steroidal anti-inflammatory drugs (NSAIDs) to manage the pain related to fractures has, however, been associated with delayed fracture healing presumably due to their effect on the cyclooxygenase (COX) pathway. Osteopontin and Osteocalcin are seldom studied in the context of fracture healing with the use of NSAIDs. Selective COX inhibitors like celecoxib may have a more profound slowing of the fracture healing process when compared to the non-selective molecules including diclofenac. The effect of NSAIDs may be more pronounced in the early fracture healing period when inflammation occurs. Altering the timing of NSAID use by avoiding them in this period may result in differences in fracture healing. The process of aging is also associated with a reduction in healing capacity and a slowing of the fracture healing process.

Objective: This study sought to determine the histomorphometry of the rat tibia fracture callus with the use of celecoxib and diclofenac while also investigating the effect of their administration at different timings and the effect of aging. The distribution of osteopontin and osteocalcin in the healing fracture callus was also investigated.

Materials and Methods: The study utilized male rats (*Rattus norvegicus*) which were divided into two groups. One group received 5mg/kg/day of diclofenac while the second group got a similar dosage of celecoxib. The animals were further divided into two sub-groups; one to receive their study medication on the day after fracture induction (early) and the other group to get their medication from the eighth day (late). A further division allocated animals to either young (5 months) or old (15 months) groups. With three animals in each group providing

baseline data on day 7, five randomly selected animals from each sub-group were euthanized on day 21 and the rest on day 42. Ethical approval was obtained; the animals were kept in standardized conditions and animal care guidelines were followed throughout the study period. The healing tibia specimens were harvested and processed for light microscopy and immunohistochemistry. After photography, the morphology of the fracture callus was described and the histological grade determined. Imaging software was used to determine the proportions of cartilage and bone. Stereological techniques were used to determine the numeric density of cells and immunohistochemistry was utilized to demonstrate the distribution of osteocalcin and osteopontin. Data was collected using MS Excel before collating and analyzed using SPSS v17.0. After determining the normality of data, the student T-test was used for pair-wise comparisons with the one-way ANOVA utilized for multivariate analysis. The Tukey HSD was used for post hoc analysis with the p set at 0.05.

Results: On day 7 the fracture callus was composed mainly of mesenchymal fibrous tissue with little cartilage. While on day 21 the fracture callus was composed of almost equal proportions of mesenchymal fibrous tissue, cartilage, and bone, by day 42 most of this had progressed to mainly bone. Over the same period, the histological grade, the proportion of bone, and the osteoblast density increased while the proportion of cartilage reduced. The celecoxib group's fracture callus was characterized by mesenchymal fibrous tissue and cartilage while the diclofenac group was composed mainly of bone and cartilage. The diclofenac group had a higher proportion of bone on day 21 (37.08% v 29.80%, p=0.021). The diclofenac group also had higher histological grades (7.20 v 6.10, p=0.004) and proportion of bone (56.71% v 46.45%, p=0.001) than the celecoxib group on day 42. The cell densities did not vary based on the drug administered on both days. Animals that received medication early had more immature fracture calluses characterized by

fibrous tissue and cartilage. The early group also had a lower proportion of bone on day 42 (47.68% v 55.47%, p=0.017). Older rats showed slower healing with the persistence of cartilage and less bone when compared to the younger ones. The older group also had a lower proportion of bone (47.68% v 55.47%, p=0.017) on day 42. Osteocalcin was expressed in the areas of immature bone with better staining in the younger animals. Osteopontin was localized in bone with robust expression in osteoblasts and osteoclasts.

When the different NSAIDs and the different timings were analyzed using multivariate analysis, the use of diclofenac late resulted in higher proportions of bone in the young (p=0.003) and older animals (p=0.049). Analyzing the different drugs in the different age groups; the young diclofenac group had the highest histological grade (p=0.004) and proportion of bone (p=0.003).

Conclusion: The use of celecoxib resulted in slowed fracture healing when compared to diclofenac. This was more evident when it was administered in the first week after a fracture and in the older animals. The use of celecoxib especially in the first week after a fracture resulted in a more cellular fracture callus which would precipitate poor biomechanics. Caution is urged when using selective COX-2 inhibitors after fractures in aging individuals.

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Fractures are a common cause of mortality and morbidity, especially in low-income countries including sub-Saharan Africa (Cordero et al., 2020). This is associated with the increase in motorization of transport coupled with improper road use contributing to an increase in the number of road traffic crashes (Jamison et al., 1999). The sub-Saharan region has also seen a rapid increase in the mechanization of the workplace and urbanization all of which have led to an increase in long bone fractures including the tibia (Streatfield et al., 2014). The tibia has also been shown to be one of the most commonly injured bones in high-energy trauma whose management is essential for the patient to resume activities of daily living (Kigera and Naddumba, 2010, Zheng et al., 2021).

Fracture healing is a regenerative process that restores the pre-injury cell composition, structure, and biomechanical function (Einhorn and Gerstenfeld, 2015). In the absence of rigid fixation, this healing goes through several stages guided by a complex interplay of various factors (Gerstenfeld et al., 2003, Marsell and Einhorn, 2011). The early stages of fracture healing involve the inflammatory pathway characterized by the production of prostaglandins via the cyclooxygenase (COX) pathway (Shapiro, 2008). This inflammatory stage is important for the mobilization and activation of the various cell components that eventually lay down the matrix that restores the structural integrity of bone (Simon et al., 2002, Zhang et al., 2002).

Fractures are associated with pain from the injury and its management which requires the use of analgesics. Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used analgesics and they work by inhibiting the COX enzyme which reduces prostaglandin production (Brune and Patrignani, 2015). NSAIDs can be classified according to their

inhibition of the two isoenzymes of COX. The COX-2 isoenzyme is the one responsible for the inflammatory pathway while the COX-1 is found in almost all cells and regulates normal cell functions (Vane and Botting, 1996). Non-selective or traditional COX inhibitors have an almost equal effect on both isoenzymes while COX-2 selective inhibitors are more selective for the COX-2 isoenzyme. Whereas celecoxib is a selective COX-2 inhibitor, diclofenac, one of the traditional NSAIDs has also been shown to preferentially inhibit COX-2 albeit to a lesser extent, and is considered a non-selective inhibitor (Kato et al., 2001). The inhibition of the COX enzyme has been shown to retard fracture healing resulting in delayed and non-union (Kurmis et al., 2012, Vuolteenaho et al., 2008)

The interaction between NSAIDs and the process of fracture healing likely takes place in the early fracture healing period resulting in a reduction in inflammatory and reparative cells and a diminution of the inflammatory response (Gerstenfeld et al., 2003). Avoiding NSAIDs in this period may likely reduce the adverse effects they have on fracture healing.

While the population of Africa is relatively young, it is rapidly aging resulting in increased numbers of older adults who may be prone to fractures(United Nations, 2019). The process of aging is associated with a reduced capacity to heal due to reduced cell proliferation and reduced expression of the COX enzyme (Lopas et al., 2014, Yukata et al., 2014). The effect of NSAIDs on fracture healing in the older population may likely be greater than in young people necessitating more judicious use of analgesics in this group of patients.

Whereas it seems clear that NSAIDs are detrimental to fracture healing, individual molecules exhibit unique properties and each molecule deserves to be investigated. It is also unclear what would be the effect of avoiding NSAIDs in the early fracture healing period. It is also unclear if young and older individuals would respond differently to this intervention.

Conducting a study to determine the effect of altering the timing of NSAID use and aging on fracture callus morphology may help shed more light on this matter.

The use of an animal model for this study is justified so that investigators have ample evidence before proposing any human studies. The benefits of the study outweigh the suffering of the animals. A recent review cemented the rat model as ideal for fracture studies (Kurmis et al., 2012). The rat model is also inexpensive, and easy to manage with the short lifespan allowing for the investigation of the influence of aging on the healing of fractures (Gomes and Fernandes, 2011). In animal models, various methods of determining fracture healing include routine histology and immunohistochemistry (IHC) (Gomes and Fernandes, 2011). These techniques are complementary and can be used in the same study to better evaluate the healing process. The drug dosages used in animal studies are determined from the pharmacokinetic studies of the molecules in rats (Kim et al., 2006, Paulson et al., 2000). The dosages are also calculated to result in almost similar plasma drug levels as is seen in humans after administration of recommended drug dosages (Reagan-Shaw et al., 2008). Diclofenac and celecoxib are commonly used drugs in orthopedic practice and it is important to know their effects on fracture healing. Though there exists some studies on the effects of these drugs on some aspects of fracture healing, information on their effect in the setting of aging and altered timings of drug administration is unknown. The aim of this study, therefore, was to determine the relationship between the timing of NSAID use and the morphology of fracture callus in young and older individuals in a rat model using celecoxib and diclofenac.

CHAPTER 2

LITERATURE REVIEW AND RESEARCH OBJECTIVES

2.1 LITERATURE REVIEW

Fracture healing goes through several stages (Sathyendra and Darowish, 2013) and the initial inflammatory stage is influenced by NSAIDs (Kurmis et al., 2012). The effect of these drugs is evidenced by their interference in the tissue composition of fracture callus (Brown et al., 2004) and the number of reparative cells (Diaz-Rodriguez et al., 2012). The effect of NSAIDs on fracture callus may be influenced by the timing of administration of the drug and the age of the individual.

2.1.1 Fracture Healing

Fracture healing is a regenerative process that restores the pre-injury cell composition, structure, and biomechanical function of bone (Einhorn and Gerstenfeld, 2015). The process of fracture healing is regulated by a complex interplay of various factors including growth factors and bone morphogenetic proteins (Sathyendra and Darowish, 2013). Fracture healing goes through several phases including the inflammatory phase, formation of cartilaginous callus, mineralization, bone formation, and remodeling (Einhorn and Gerstenfeld, 2015, Bahney et al., 2019). In fractures stabilized with an intramedullary device, complete rigidity is not possible and the fracture heals by secondary healing that involves both intramembranous and endochondral ossification (Loi et al., 2016). The initial processes of fracture healing involve an inflammatory phase (Shapiro, 2008) which lasts about seven days and is regulated by several cytokines (Gerstenfeld et al., 2003, Marsell and Einhorn, 2011). These cytokines play a role in the mobilization of mesenchymal stem cells (MSCs) which are later transformed into chondrocytes in the fracture callus (Pajarinen et al., 2019). It has been established that prostaglandins are vital to the functioning of osteoblasts and osteoclasts in

the fracture callus (Kurmis et al., 2012, Simon and O'Connor, 2007, Simon et al., 2002). Because they inhibit the inflammatory processes, the effect of different NSAIDs on fracture healing thus deserves further investigation.

In a rat fracture model, by day 21 we expect exuberant callus with cartilage and newly formed bone while by day 42 we expect maturing bone with little cartilage remaining (Kokubu et al., 2003). Non union models demonstrate that the callus is manly cartilage at day 21 and immature bone at day 42 (Kokubu et al., 2003). The histological grade is expected to be about 5.5 by day 21 and 7.0 by day 42 (Dincel et al., 2018).

2.1.2 Use of NSAIDs in Fracture Management

NSAIDs are commonly prescribed to manage the pain associated with fractures (Griffioen and O'Brien, 2018). Analgesics including NSAIDs are the most common over-the-counter (OTC) medication consumed by both young and aging adults (Kiza et al., 2021, Paliwal et al., 2021). Both diclofenac and celecoxib are freely used and available as OTC medications in many parts of the world (Netere et al., 2018, Losina et al., 2018). They are also an integral part of multimodal pain management which attempts to harness the efficacy of different molecules while reducing the possibility of side effects (Hsu et al., 2019). NSAIDs especially diclofenac are also commonly used in the perioperative setting and have been shown to reduce the need for opioids (Souter et al., 1994). Diclofenac has been found to be one of the most efficacious molecules in the perioperative management of lower limb fractures (Griffioen and O'Brien, 2018). Celecoxib has been shown to be similar to non selective COX inhibitors in pain management in the acute setting (Salo et al., 2003). Diclofenac and Celecoxib are efficacious in acute musculoskeletal injuries. They are also widely available and used in orthopedic practice making them ideal candidates in this study.

2.1.3 Histomorphology and Histomorphometry of Fracture Callus with the use of NSAIDs

NSAIDs have been found to have varying effects on the tissue proportions in the healing fracture callus. The use of various selective COX-2 inhibitors has been associated with the persistence of fibrous tissue and cartilage in the fracture callus. The use of rofecoxib resulted in more fibrous tissue and cartilage than bone (Murnaghan et al., 2006, O'Connor et al., 2009). Celecoxib was associated with more fibrous tissue and less woven bone in the early stages of fracture healing (Brown et al., 2004). It has also been associated with reduced histological scores through the inhibition of the chondrogenic phase of fracture healing (Janssen et al., 2017). Valdecoxib was associated with more cartilage formation in the early stages of fracture healing (Gerstenfeld et al., 2007). COX-2 inhibitors were also associated with poor mineralization of the fracture callus. The use of rofecoxib resulted in poor mineralization and less woven bone formation (Goodman et al., 2005).

Non-selective NSAIDs are also associated with impaired healing of fractures. The histological scores of healing fractures in animals given diclofenac have been shown to be similar to placebo and better than meloxicam (Inal et al., 2014). Another study found that the number of specimen with bone present in the healing fracture callus was reduced in animals given diclofenac when compared with prednisone and placebo (Bissinger et al., 2016).

The use of indomethacin was associated with more fibrous tissue (Brown et al., 2004, Altman et al., 1995). Ibuprofen was also associated with a less mature callus when compared to a

placebo (Altman et al., 1995). While both selective and non-selective NSAIDs have been associated with impaired fracture healing, several studies did not show any changes to the fracture callus with the use of some NSAIDs. The use of dexketoprofen in a rat fracture model did not show any differences in the fracture callus compared to a placebo over six weeks (Sevimli et al., 2013). Ketorolac use was also not associated with any changes in the fracture callus (Cappello et al., 2013). Different studies have also shown varying results with the same molecule. While ibuprofen was also associated with a less mature callus when compared to placebo in one study(Altman et al., 1995), another study showed no difference in the fracture callus healing (O'Connor et al., 2009).

There is therefore controversy on the effect of different NSAIDs on fracture callus and hence the need to investigate each molecule to determine its effect. A recent systematic review suggested that NSAIDs should be used with caution, but did not explore the issues of the type of COX inhibition, the timing of drug administration and the effect of aging (Al-Waeli et al., 2021). While there are a few studies on the effect of celecoxib, they mainly compare the drug to placebo and the histomorphometric data is limited. The few studies on diclofenac found in literature were of a short duration and did not measure the different tissue proportions in the healing callus. We came across no studies comparing the histological grades and tissue proportions in healing fracture callus after the use of diclofenac and celecoxib, commonly used NSAIDs in orthopedics. There is hence a need for more data on the subject.

2.1.4 Stereology of Cell Cultures and Fracture Callus with the use of NSAIDs

Various NSAIDs have resulted in altered numbers and morphology of various cells found in bone and fracture callus. Various non-selective NSAIDs have been associated with varied effects on the cells. Indomethacin was associated with increased apoptosis of osteoblasts in culture (Liu et al., 2012). Indomethacin was also been implicated in reduced osteoblast proliferation (Diaz-Rodriguez et al., 2012). Ketorolac use in juvenile rats, however, did not affect the number or morphology of chondrocytes in the fracture callus when compared to placebo (Cappello et al., 2013). Diclofenac use resulted in reduced osteoblast proliferation (Diaz-Rodriguez et al., 2012). Diclofenac was found to induce more apoptosis of chondrocytes taken from patients with osteoarthritis than celecoxib (Nakamura et al., 2007). Selective COX-2 inhibitors have also been shown to affect the cells in cell cultures and fracture callus. Celecoxib was implicated in the reduction of apoptosis of chondrocytes in cell culture (Jeffrey and Aspden, 2007). Rofecoxib use was associated with reduced numbers of osteoclasts and osteoblasts (Goodman et al., 2005). The use of Valdecoxib is also associated with reduced cell elements and bone lining cells in the fracture callus (Gerstenfeld et al., 2007). The effects of NSAIDs on various cells in fracture callus are varied across the molecules studied hence the need to expound on the role of each different molecule. While there is some limited data on diclofenac, there is a paucity of studies on celecoxib. The majority of the studies in the literature also deal with the effects of NSAIDs on cells in a culture medium with very few addressing the stereology of fracture callus. There is a need to further elucidate this subject.

2.1.5 Changes in Fracture Healing with Different Timings of NSAID Use

Several authors seem to suggest that the use of NSAIDs in the early period of fracture repair is detrimental to the healing process. A recent systematic review highlighted that the use of NSAIDs in the early stages of fracture repair in animal models may result in reduced mechanical properties of the fracture callus (Kurmis et al., 2012). Some non-selective COX inhibitors have been investigated in this regard. While a study showed that early administration of ketorolac in a rabbit model showed impaired fracture healing (Ho et al., 1998), a clinical study did not show any adverse effects with the early administration of ketorolac (Donohue et al., 2016). Another clinical study showed that the use of indomethacin for six weeks resulted in poor healing while its use for one week did not adversely affect fracture healing (Sagi et al., 2014). This study however muddles the issues of the timing of the drug and the duration of use.

Selective COX-2 inhibitors similarly have been shown to have some effects on fracture healing. Avoiding the use of celecoxib in the early period of fracture repair was not associated with the alteration of the mechanical properties of fracture callus (Simon and O'Connor, 2007). Early administration of rofecoxib inhibitor did not impair fracture healing in skeletally mature rabbits (Hak et al., 2011). There is limited data on the effect of altering the timing of NSAID use on fracture healing. It is also unclear if this effect is similar across the various NSAIDs raising the need to investigate each molecule.

2.1.6 Changes in Fracture Healing with Aging

The global human population is aging and this age group constitutes a large proportion of fracture patients (United Nations, 2019). Aging is associated with a reduced healing response including a reduction in the amount of callus produced (Lopas et al., 2014, Clark et al., 2017). The reduced healing response may be associated with the decreased proliferation and differentiation of the osteoprogenitor cells found on the surface of bones (Yukata et al., 2014). The reduced healing may also be due to a diminution of the inflammation seen in the early fracture healing period. Animal studies have shown reduced expression of COX-2 in aging and this was associated with reduced healing response (Naik et al., 2009). The fracture callus in aged animals shows unregulated inflammation and reduced cell proliferation (Hebb et al., 2018). The use of COX inhibitors may likely worsen this situation.

A study on juvenile rats did not show any impairment of fracture healing when ketorolac was compared to a placebo (Cappello et al., 2013). In contrast, a systematic review shows that the use of NSAIDs in the older population is associated with a higher incidence of non-unions. Long-term use of analgesics in the elderly was also shown to reduce the capacity of bone to heal (Kurmis et al., 2012). There may be a relationship between the adverse effects of NSAIDs on fracture healing and increasing age. To determine whether this should be expected with different analgesics, further investigation is warranted.

2.1.7 Role of Osteopontin and Osteocalcin in Fracture Healing

Osteopontin and osteocalcin are non-collagenous proteins found in bone with the former belonging to the glycoprotein class while the latter is a gamma carboxyglutamic acid derivative (Bonucci, 2012). Osteopontin is involved in fracture healing and may play a role in

the formation of callus and its remodeling (Duvall et al., 2007). Osteocalcin has been implicated in the laying down of new bone and its mineralization (Berezovska et al., 2019). These molecules are vital in fracture healing but they are seldom investigated in this context. Staining for them in the fracture callus could shed more light on their role in the various stages of fracture healing in different individuals with the use of NSAIDs.

2.2 STATEMENT OF THE PROBLEM, JUSTIFICATION, AND SIGNIFICANCE

2.2.1 Problem Statement

Fractures and their management are associated with pain for which analgesics are indicated. NSAIDs are frequently used in this setting but they work by inhibiting the COX enzyme which reduces prostaglandin production (Brune and Patrignani, 2015). This effect may alter the healing of fractures. Based on their inhibition of the COX enzyme, celecoxib is a selective COX-2 inhibitor while diclofenac is considered a non-selective inhibitor (Kato et al., 2001). The action of NSAIDs happens in the early fracture healing period causing a reduction in inflammatory and reparative cells and a diminution of the inflammatory response (Gerstenfeld et al., 2003). Aging on the other hand, is associated with a reduced capacity to heal due to reduced cell proliferation and reduced expression of the COX enzyme (Lopas et al., 2014, Yukata et al., 2014). NSAIDs are an integral component of most analgesic regimes and while they have been shown to retard fracture healing, it is unclear the effect altering the timing of their use may have. It is also unclear if this effect may differ among young and older individuals.

2.2.2 Study Justification

The incidence of long bone fractures has been on the rise worldwide and in the sub-Saharan region (Jamison et al., 1999). As a result of better nutrition and healthcare, life expectancy in the sub-Saharan region is increasing (WHO, 2015). This would increase the number of older patients who may suffer from long bone fractures. NSAIDs are commonly prescribed to

manage the pain associated with fractures (Griffioen and O'Brien, 2018). NSAIDs are part of multimodal pain management (Hsu et al., 2019). Analgesics including NSAIDs are the most common over-the-counter (OTC) medication consumed by both young and aging adults (Kiza et al., 2021, Paliwal et al., 2021). Both diclofenac and celecoxib are freely used and available as OTC medications in many parts of the world (Netere et al., 2018, Losina et al., 2018). The increase in the aging population and the rampant use of NSAIDs may increase the number of patients with fractures that fail to unite. The morbidity and economic costs of this would be great. The potential human and economic savings by identifying particular NSAIDs that should be avoided during fracture management would be enormous.

2.2.3 Significance of the Study

NSAIDs are associated with a delay in the process of fracture healing leading to the development of delayed union and non-union (Kurmis et al., 2012). This may result in increased morbidity and costs to the patient. It may also lead to the need for additional surgical procedures (Patil and Montgomery, 2006). The role of avoiding NSAIDs in the early fracture healing period is unclear and it is also unclear if there are any differences in the response of younger and older individuals to this intervention. The results of this study will provide evidence on the role of NSAIDs in the early stages of fracture healing and the effect of aging on this. This will aid the clinician in determining the place of NSAIDs in the management of fractures and the choice of NSAIDs for different age groups.

2.2.4 Conceptual Framework

The effect of NSAIDs on fracture healing is most likely a result of disruption of the early stages of inflammation by inhibition of the COX enzyme. It is also likely that this effect is greatest in the early phase of fracture healing. We propose that avoiding NSAIDs in this early

fracture healing period may reduce their detrimental effect on fracture healing. We further propose that there is a difference in the response of older and younger individuals to this intervention (Figure 1).

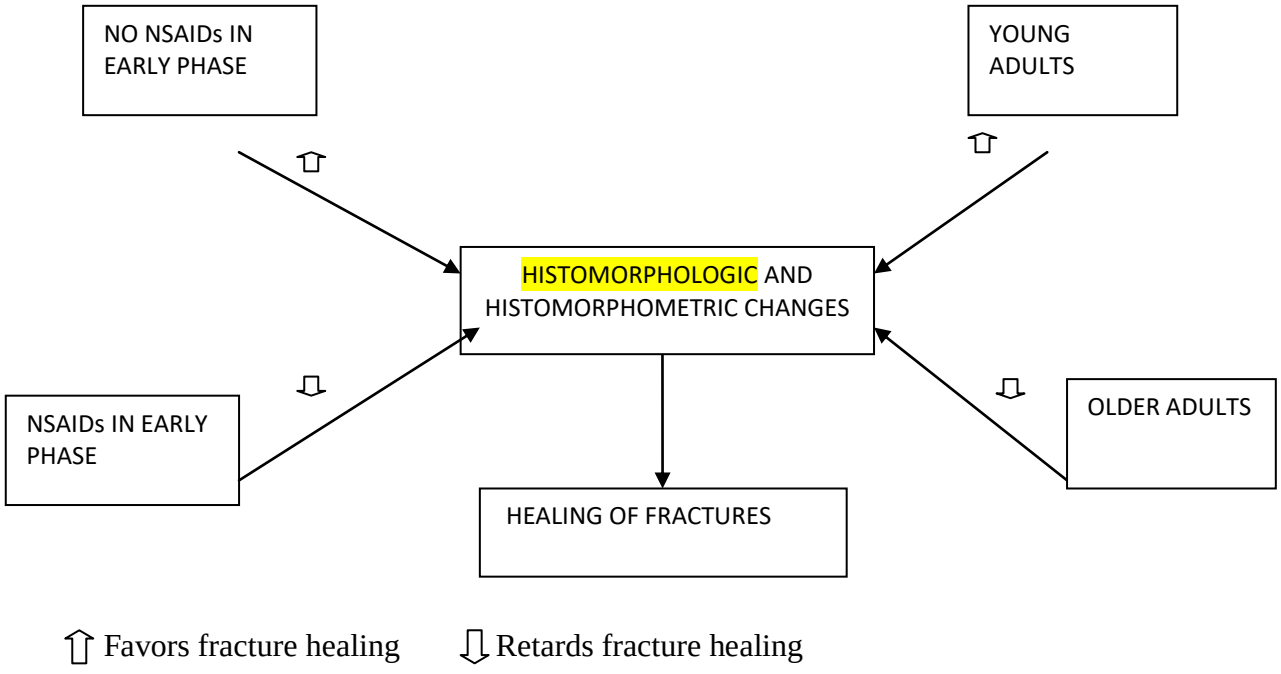


Figure 1. Conceptual framework

2.3 RESEARCH QUESTION, HYPOTHESIS, AND OBJECTIVES

2.3.1 Research Questions

1. Is the effect of NSAIDs on fracture callus morphology and morphometry influenced by the drug?
2. What would be the effect of avoiding NSAIDs in the first week after a fracture?
3. Is there a difference in how fractures of young and old individuals heal after the

administration of Diclofenac and Celecoxib?

2.3.2 Study Hypotheses

Null Hypotheses -

1. Fracture callus histomorphology and histomorphometry are not influenced by the drug used
2. The timing of NSAID use has no impact on the histomorphology and histomorphometry of fracture callus
3. The age of the individual has no impact on the histomorphology and histomorphometry of the healing fracture callus

2.3.3 Study Objectives

2.3.3.1 Broad Objective

To determine the effect of timing of NSAID use and aging on fracture callus histomorphology and histomorphometry

2.3.3.2 Specific Objectives

About the rat tibial fracture callus model using two different analgesics;

1. To compare the histomorphology and histomorphometry of fracture callus with the use of diclofenac and celecoxib
2. To determine the differences in the histomorphology and histomorphometry of the fracture callus with the use of NSAIDs at different timings
3. To evaluate the relationship between aging and histomorphology and histomorphometry of fracture callus with the use of NSAIDs
4. To describe the distribution of osteocalcin and osteopontin in fracture callus after the use of NSAIDs

CHAPTER 3

MATERIALS AND METHODS

3.1 STUDY DESIGN AND SAMPLE SIZE

3.1.1 Study Design

This was a randomized experimental study where animals were randomly allocated to the study groups. Preparation of the study medication and surgical procedures were performed by the principal investigator. The research assistants who handled the animals, administered the drug medication and harvested tissue specimens were unaware of the study groups of the animals. The research assistants who assisted in evaluation of the histological grade, tissue proportions and cellular densities were also blinded to the group allocation.

3.1.2 Sample Size

This was an experimental study that compared means of continuous data between the groups studied. For this reason, the formula below was used

$N=1+2C (\sigma/D) 2$, And $C= (Zcrit+Zpwr) 2$ (Snedecor and Cochran, 1989), Where N is the desired sample size per group, σ is the standard deviation of each group, D is the smallest meaningful difference expected and Zcrit and Zpwr are constants determined by the specified significance criterion (0.05 for this study) and the desired statistical power (0.8 for this study) respectively.

A previous study on the effect of NSAIDs on fracture callus histomorphometry revealed that the σ for the histological grades was 3.2 and the difference seen between groups was 5.67 (Janssen et al., 2017). The calculated sample size was 5 animals per group. To ensure measurement on both days 21 and 42, this was doubled to 10, bringing the total sample size for the study to 80 animals.

3.2 MATERIALS

3.2.1 Rat Model

Five-month and fifteen-month-old male Norwegian rats (Rattus norvegicus), were obtained from the department of biochemistry at the University of Nairobi. These ages were been chosen as they correspond to 18 and 50 human years (Sengupta, 2013). In the laboratory, the animals were kept in pens floored with saw dust, at room temperature and humidity, and on a 12-hour light /dark cycle. They were fed on commercial pellets and given a steady supply of fresh water. The rats were acclimatized to the study environment, other animals, and handling before commencing the study. Animals were weighed daily, before conducting the experiments, and at euthanasia.

3.2.1.1 Inclusion and Exclusion Criteria

The study included animals of the specified ages that had no gross musculoskeletal abnormalities. Animals that suffered comminuted or open fractures and those that developed post-operative infections were excluded and replaced. Animals that required rescue analgesia and those that suffered other injuries other than a left tibial fracture were also excluded from the study and replaced.

3.2.1.2 Selection

Qualifying animals of both age groups were first divided into two groups with the first group receiving diclofenac and the second celecoxib. The animals in each group were further subdivided into those to receive no medication but saline only in the first week and then NSAID for the next three weeks (late). The other subgroup received the chosen NSAID from

the day after fracture induction for four weeks (early). The animals were further divided into young (five months) and old animals (fifteen months). The animals were then allocated numbers and then randomly selected to any of the groups. All animals were subjected to fractures of the left tibia which were fixed with an intramedullary wire. Five animals from each sub-group were euthanized on days 21 and 42 (Kokubu et al., 2003). Three animals from each group were used to provide baseline data.

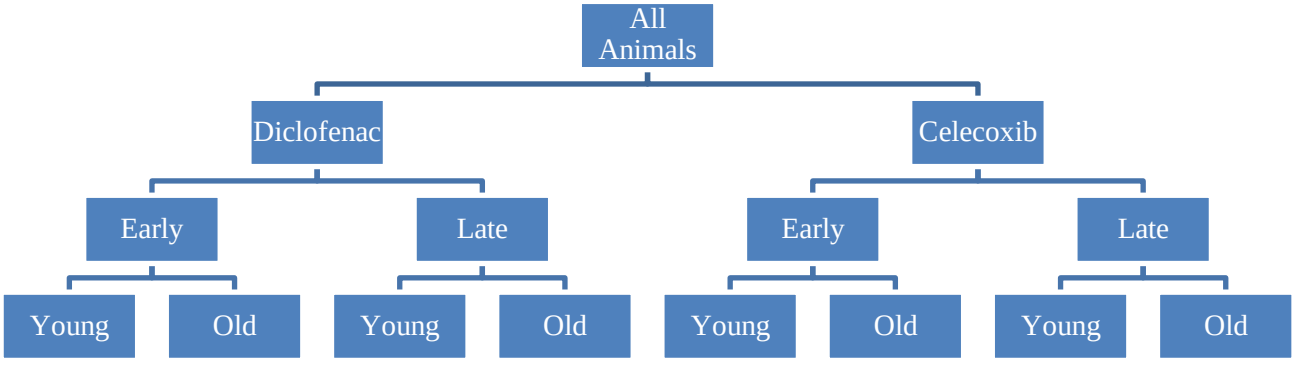


Figure 2. Distribution of study animals

3.2.2 Surgical Procedures

3.2.2.1 Anesthesia and Kirshner Wire Fixation

The animals were anesthetized before all surgical procedures using ketamine 100mg/kg and xylazine 10mg/kg IP with the direction of a qualified veterinarian. The surgical area was shaved and cleaned with iodine in alcohol. The tibia was fixed with an intramedullary 1.25mm Kirshner wire inserted from the knee joint under sterile conditions (Kokubu et al., 2003).

3.2.2.2 Fracture Induction

The left lower limb was held in a fixed position of abduction and external rotation while a weight (460 g) was dropped from a height of 20cm to produce a fixed traumatic injury in the middle of the bone, leading to a closed, transverse fracture via three-point bending (Yang et al., 2012) (Figure 3). After the operation, a sciatic nerve block of the ipsilateral limb was conducted using bupivacaine 0.5% to provide prolonged postoperative analgesia (Thalhammer et al., 1995, McAlvin et al., 2014). The animals were monitored every half hour until they awoke from anesthesia and were permitted full weight-bearing and unrestricted movement thereafter. In the post-surgical period, animals were assessed for pain and any other postoperative complications. Animals not responding to the analgesia given were given rescue analgesia after exhibiting the signs in the first stage of the criteria set out (Roughan and Flecknell, 2001, Ciuffreda et al., 2014)(Appendix 1).

Figure 3. Device for inducing fractures

3.2.3 Administration of Therapeutic Medication

The animals received NSAIDs as per the groups they are allocated to using a gavage designed for the purpose. Half the animals received diclofenac 5mg/kg/day (Novartis Pharma AG, Basel Switzerland) while the others received celecoxib 5mg/kg/day (Pfeizer Pharmaceuticals LLC, Illertissen, Germany). Both drugs were administered in two divided doses twelve hours apart.

3.2.4 Specimen Collection

At the selected intervals, five animals from each sub-group were selected randomly and euthanized. Euthanasia was accomplished by inhalation of 3% halothane in an airtight glass chamber until the animals had no pupillary reflexes and little response to pain. The animal was subsequently opened through a mid-line abdominal incision and a thoracotomy was performed to expose the heart. For complete clearance of blood, a cannula was inserted into the left ventricle and buffered normal saline (0.2L/Kg) introduced from a perfusion kit 1.5 meters above the heart followed by buffered 10% formaldehyde solution perfused for 30 minutes. After fixation, the left lower limb of the animal was disarticulated and soft tissue dissected off while preserving the periosteum. The intramedullary wire was removed and the specimen was weighed and the volume determined using Scherle's method (Weibel et al., 1966). The specimen was then stored in 10% formal saline for 48 hours.

3.2.5 Decalcification

The specimen underwent decalcification in 10% ethylenediaminetetraacetic acid (EDTA) until the end point was achieved. The endpoint of decalcification was determined using the flexibility and calcium oxalate methods (Callis and Bancroft, 2008).

3.2.6 Occupational Health

The animals were handled by trained research assistants. Bites from the animals were avoided by wearing bite-resistant gloves when handling the animals. With the non-dominant hand, the base of the tail was held with the thumb and index finger to keep the rat from running away when transferring them from one place to another, feeding, and cleaning. When carrying out oral NSAID administration, the rat was grasped with firm but gentle pressure around the thorax with the thumb and fingers under each of the front legs. The thumb was used to hold firm the forelegs to allow for greater control and manipulation of the head. This reduced chances of rat bites during handling.

Any wounds inflicted by the animals on the personnel were immediately disinfected. Minor wounds were disinfected using 70% alcohol and bandaged while medical attention was sought for more serious wounds. All sharps used in the study were disposed of in sharps bottles. The euthanized animal carcasses were incinerated at the incinerator at the Chiromo campus.

Protective gear including lab coats, sterile latex gloves, masks, and goggles was worn during fixing and tissue processing reducing the chances of getting in direct contact with the laboratory chemicals. Tissue processing was performed in a fume cupboard in a well-ventilated laboratory in the Department of Human Anatomy, University of Nairobi.

3.2.7 Ethical Considerations

All procedures were performed according to the Guide for Care and Use of Laboratory Animals (Eighth edition, 2011) and guidelines of the Biosafety, Animal Use and Ethics Committee, Faculty of Veterinary Medicine, the University of Nairobi where ethical approval was sought and obtained under reference FVM BAUEC/2016/92.

3.3 DETERMINING THE HISTOMORPHOMETRY OF FRACTURE CALLUS

3.3.1 Processing for Light Microscopy

After decalcification, the tibia was trimmed to isolate the fracture callus area which was processed in one block. These specimen were then dehydrated in increasing concentrations of alcohol and prepared for embedding using Xylol immersion. The tissues were then embedded in paraffin wax at 70^{o}C for 12 hours. Seven-micrometer sections in the sagittal plane were taken, floated in warm water, and thereafter mounted and then dried in a hot air oven at 40^{o}C overnight. Haematoxylin and eosin (H&E) stain was used to demonstrate the fracture callus morphology while Masson's Trichrome (MT) was used to highlight the matrix.

3.3.2 Photography

Five randomly selected slides from each block were chosen for photography. Photomicrographs were taken using a Motic BTU8 (Motic, Kowloon, Hong Kong) digital camera mounted on a Richter Optica UT1 microscope (Richter Optica, Kowloon, Hong Kong). Five randomly selected images from each slide were taken and analyzed using the Image J software (NIH, Massachusetts, USA).

3.3.3 Histomorphology, Histomorphometry, and Stereology

Images taken at X100 magnification were used to describe the morphology of the fracture callus across all the groups. The same images were used for determining the histological grade using an objective scale that allocates 1 for fibrous tissue and 10 for mature bone (Huo MH et al., 1991) (Appendix 2).

The imaging software was utilized to superimpose a grid on the chosen images at X100 for the evaluation of tissue densities. The cortical bone from the fractured end was excluded from the measurement (Figure 4A). The number of points falling on the total callus area and the areas of bone and cartilage were counted and recorded with the aid of the software. All the counts were verified by the principal investigator with the use of the image software.

The imaging software was also used to superimpose a counting frame on images at X400 for the evaluation of the number of cells (Figure 4B). The sampling at the image level was done by starting at a fixed point at the top left-hand corner and moving with a constant step towards the right to count five boxes from a sample of twenty. Each box had a forbidden line (bottom and left borders) observed in each chosen box (red lines). The number of cells was counted and recorded with the aid of the software. Osteoblasts were identified as the small rounded cells in a bone matrix including those on surfaces, and with cuboidal/columnar morphology; while chondrocytes were defined as large rounded cells in lacunae in a cartilage matrix. As much as possible, the nomenclature adopted by the American society for bone and mineral research was adopted (Dempster et al., 2012).

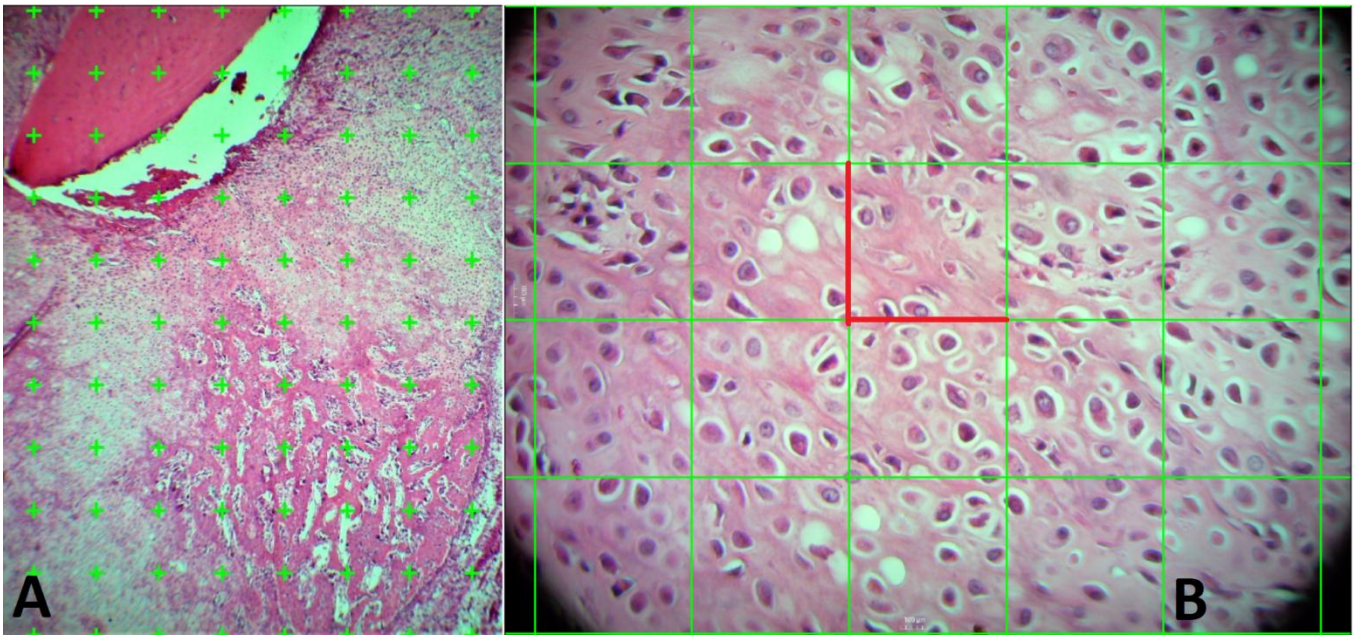


Figure 4 A-B. Estimating the volume and numerical densities

3.4 DETERMINING THE DISTRIBUTION OF OSTEOCALCIN AND OSTEOPONTIN

Immunohistochemistry aimed at identifying the distribution of osteocalcin and osteopontin in the fracture callus and was performed with reagents from Elabscience Inc (Wuhan, China) which were supplied as a kit. Four-micrometer sections in the sagittal plane were taken from the tissue block, floated in warm water, and thereafter mounted on positively charged slides and then dried in a hot air oven at 40^{o}C overnight. The slides were then baked in an oven at 65 degrees for 30 minutes before being placed in the dewaxing and antigen retrieval solution at 95 degrees for 15 minutes. After cooling naturally to room temperature the slides were washed with deionized water, running tap water, and later wash buffer solution. Peroxidase blocking buffer was added and washed off after 15 minutes. The primary antibody (osteocalcin or osteopontin) was added and the slides were incubated at 37 degrees for 40

minutes before adding the secondary antibody and incubating for 30 minutes. The DAB working solution was then added and coloration was observed after about five minutes and the reaction was terminated by washing with deionized water and wash buffer. Hematoxylin stain was added for five minutes to counterstain the nuclei. This was followed by dehydration with alcohol and cleared with xylene. The sides were dried and cover slips mounted. One slide from each block was prepared and five images were taken for each slide. The images were used to describe the distribution of the receptors across the groups.

3.5 DATA HANDLING AND STATISTICS

The average of two readings of the histological grade was entered onto a datasheet. This data and the excel output (Microsoft Corp, Washington, USA) from the image software was used to determine the averages for each slide and animal. The data was then exported to SPSS v17.0 (SPSS Inc, Chicago, USA) for analysis.

The volume density of bone and cartilage was determined using the formula $Vvtis=\sum Pttis/\sum Pttot*100$, where Pttis is the number of points falling on the tissue; Pttot is the total number of points falling on the fracture callus and Vvtis is the volume density of the tissue. The number of cells was counted using a counting frame of $5000um^2$. The numerical density of various cells was determined using the formula $Nncell=\sum Q\text{-cell}/\sum Atot$. Where Q- was the number of cells counted and Atot was the area of the counting frame. The value was then converted to cells/mm^2 for comparison across the groups (Makanya et al., 2020).

The normality of the data was determined using histograms and box plots. To determine the differences in the histomorphometric and stereological parameters between the animals that received diclofenac and celecoxib, early and late, and old and young, the data were divided

into two groups, and the parameters were compared using the student T-test. To examine the relationship between the drug used and the timing of administration, the drug and aging, and aging and the timing of administration, the data were divided into four groups. The one-way ANOVA was used to determine differences across the four groups and post hoc analysis was performed using the Tukey HSD. A p-value of <0.05 was considered significant. Histological images, tables and graphs were used to illustrate the findings.

CHAPTER 4

RESULTS

4.1 GENERAL FEATURES OF THE RAT TIBIA AND TEMPORAL CHANGES WITH THE USE OF NSAIDS

4.1.1 General Features of the Rat tibia

The tibia is the main bone of the leg and in the rat extends from the knee joint to the ankle. It is easily palpable as it is not covered by many soft tissues when compared to the femur. The bone is easily accessible for fracture induction and surgical procedures (Figure 5A). It is slightly curved anteriorly with a subcutaneous border (Figure 5B).

Figure 5A-B. Photograph of the rat hind limb and tibia

A: Rat hind limb showing the leg (white arrow).

B: Rat tibia after stripping the soft tissues and exposing the bone. Note the anterior bowing of the bone.

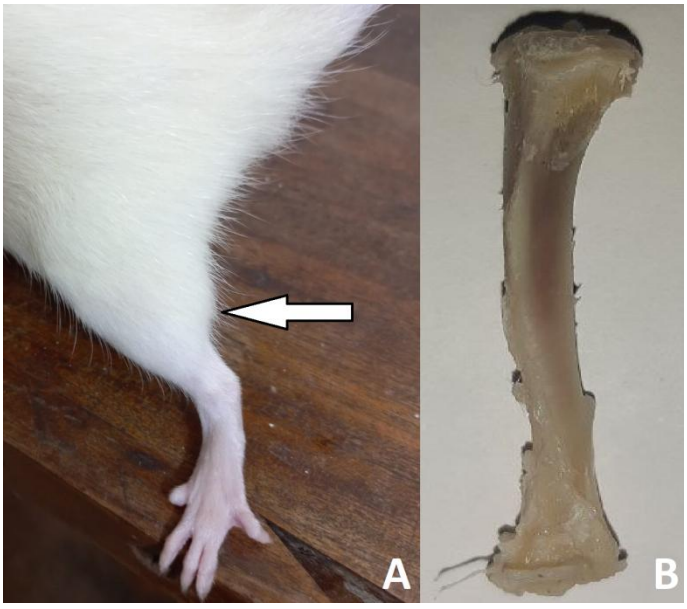


Figure 5 A-B. Photograph of the rat hind limb and tibia.

4.1.2 Temporal Changes of the Rat Tibia Fracture Callus with the use of NSAIDs

The microscopic structure over time showed progressive healing with the replacement of the mesenchymal fibrous tissue with cartilage and eventually bone. The histological grade and proportion of bone increased while the proportion of cartilage reduced as the healing progressed. While the osteoblast density increased over time, the chondrocyte density did not vary much as the tibial fracture was healing.

4.1.2.1 Histomorphology of the Temporal Changes of the Rat Tibia Fracture Callus with the use of NSAIDs

Histologically the fracture callus gradually changes from mesenchymal fibrous tissue to bone. The fractured end of the bone shows mature cortical bone with rugged fracture edges. On day 7 the fracture callus is immature with mainly mesenchymal fibrous tissue and islands of cartilage. This tissue is well vascularized with numerous blood vessels (Figure 6A). The fibrous matrix is arranged in irregular bundles with numerous flattened cells (Figure 6B). At higher magnification, the mesenchymal cells are flattened with some showing transformation to more plump cells (Figure 6C). The cartilage islands show cells in lacunae. The cells are arranged in isogenous groups and several cells are in various stages of cell division (Figure 6D).

Figure 6 A-D. Photomicrograph of the rat tibia fracture callus on day 7 in rats that received diclofenac

A: Fracture callus showing the fractured end (white diamond), cartilage (C), and mesenchymal fibrous tissue (F) [Haematoxylin and Eosin stain, X100].

B: Fracture callus showing the mesenchymal fibrous tissue with islands of cartilage. Note the numerous blood vessels (stars) in the callus [Masson's Trichrome, X100].

C: Higher magnification of the section in Figure 6A showing mesenchymal fibrous tissue. Note the numerous flattened cells (left-pointing arrow) and a blood vessel (star) [Haematoxylin and Eosin stain, X400].

D: Higher magnification of the section in Figure 6A showing cartilage with large rounded cells in lacunae. Note the large cells with prominent nuclei (right-pointing arrow) [Haematoxylin and Eosin stain, X400].

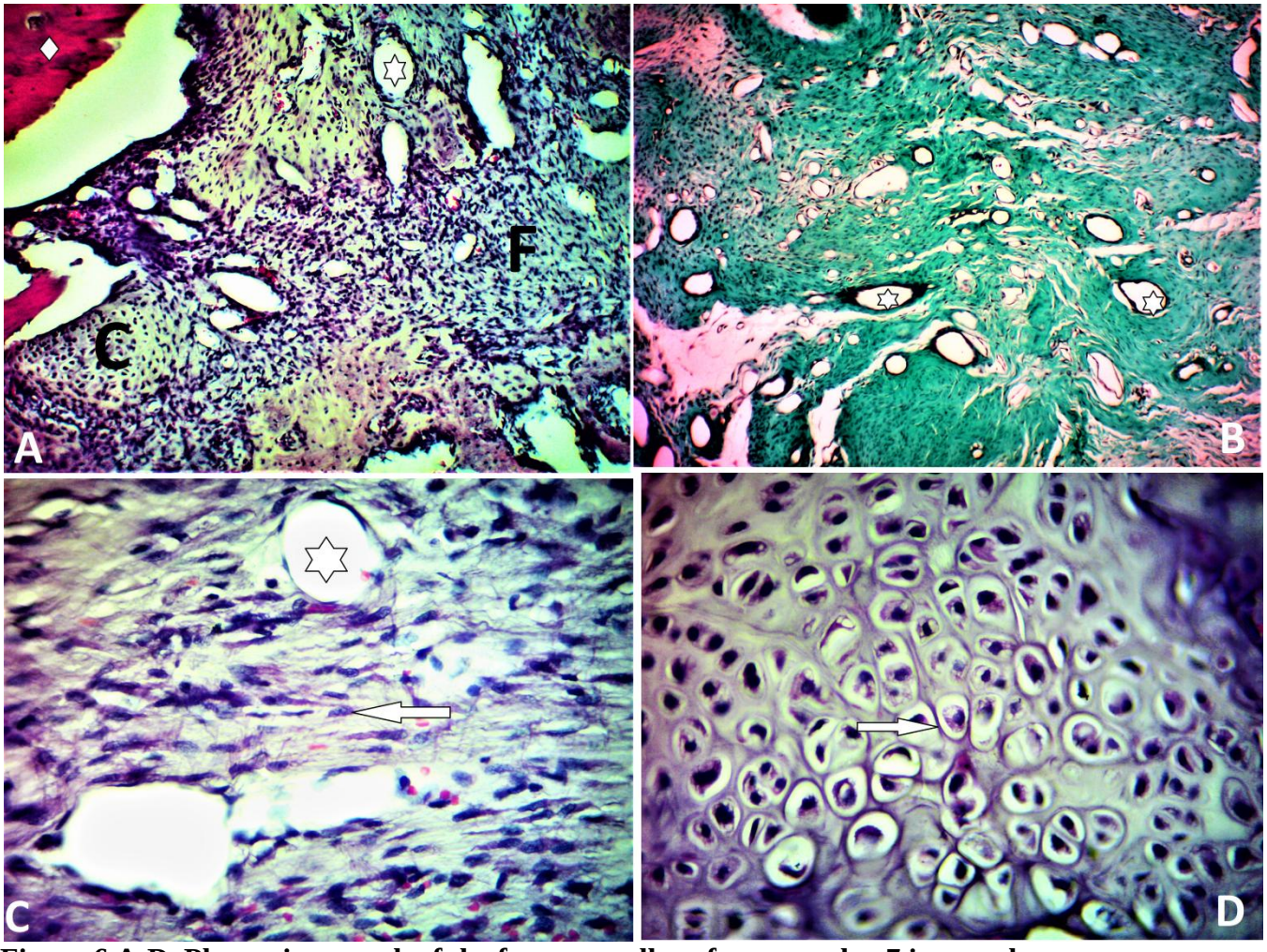


Figure 6 A-D. Photomicrograph of the fracture callus of a rat on day 7 in rats that received diclofenac

On day 21 the fracture callus shows almost equal proportions of bone, cartilage, and mesenchymal fibrous tissue. The mesenchymal and cartilage cells are closely packed with little matrix between them while bone has fewer cells and numerous vascular channels (Figure 7A). The matrix in the areas of mesenchymal fibrous tissue is made of irregularly arranged fibers while the cells are numerous and spindle-shaped. The matrix in the cartilage zones does not take up the stains well and looks clear while that in the zones with bone looks densely packed and regular (Figure 7B). At higher magnification, the areas of bone reveal small rounded cells in a matrix that has numerous vascular channels and multinucleated osteoclasts (Figure 7C). The cartilage shows larger rounded cells in lacunae. The cells are closely packed together in a plain matrix (Figure 7D). The mesenchymal fibrous tissue shows numerous closely packed spindle-shaped cells. The matrix is fibrous and devoid of vascular channels (Figure 7E).

Figure 7A-E. Photomicrograph of the rat tibia fracture callus on day 21 in rats that received diclofenac

A: Fracture callus showing the fractured end (white diamond), bone, cartilage, and mesenchymal fibrous tissue [Haematoxylin and Eosin stain, X100].

B: Fracture callus showing the bone, cartilage, and mesenchymal fibrous tissue. Note the changes in the matrix in the three zones [Masson's Trichrome, X100].

C: Higher magnification of the section in Figure 7A showing the area with bone. Notice the small rounded cells in lacunae surrounding vascular channel (stars) multinucleated osteoclasts (down-pointing arrow) [Haematoxylin and Eosin stain, X400].

D: Higher magnification of the section in Figure 7A showing cartilage with large rounded cells in lacunae in an amorphous matrix. Note the large cells with prominent nuclei (right-pointing arrow) [Haematoxylin and Eosin stain, X400].

E: Higher magnification of the section in Figure 7A showing mesenchymal fibrous tissue. Note the numerous spindle-shaped cells (left-pointing arrow) [Haematoxylin and Eosin stain, X400].

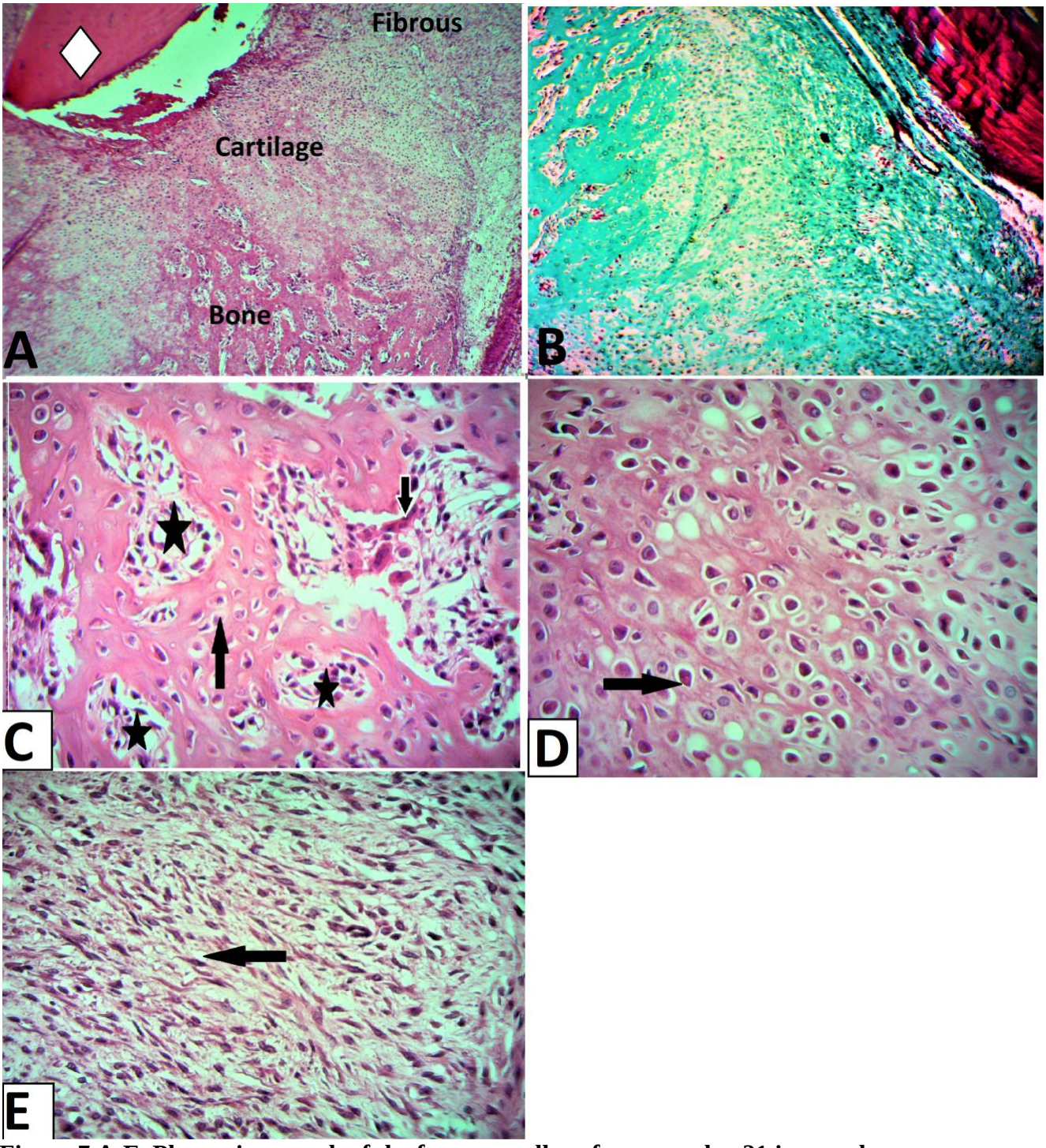


Figure 7 A-E. Photomicrograph of the fracture callus of a rat on day 21 in rats that received diclofenac

On day 42 most of the fibrous and chondroid tissue has been replaced by mature bone showing small rounded cells with scattered vascular channels of reduced size (Figure 8A). The matrix is densely packed and regularly arranged around the canals. While the territorial matrix is deeply staining, the inter-territorial matrix stains lightly (Figure 8B). At higher magnification, the bone shows Haversian canals with the matrix arranged in concentric lamellae around the canals. The number of cells has reduced with the increase in the matrix (Figure 8C).

Figure 8 A-C. Photomicrograph of the rat tibia fracture callus on day 42 in rats that received diclofenac

A: Maturing rat tibia fracture callus showing mainly bone tissue. Note the reduced vascularity of the bone with the bone tissue organized in concentric circles around a vascular channel [Haematoxylin and Eosin stain, X100].

B: Fracture callus showing relatively mature bone. Note the densely packed matrix which is regularly arranged around the vascular canals [Masson's Trichrome, X100].

C: Higher magnification views of the section in Figure 8A showing mature bone arranged in concentric circles around vascular channels (stars) [Haematoxylin and Eosin stain, X400].

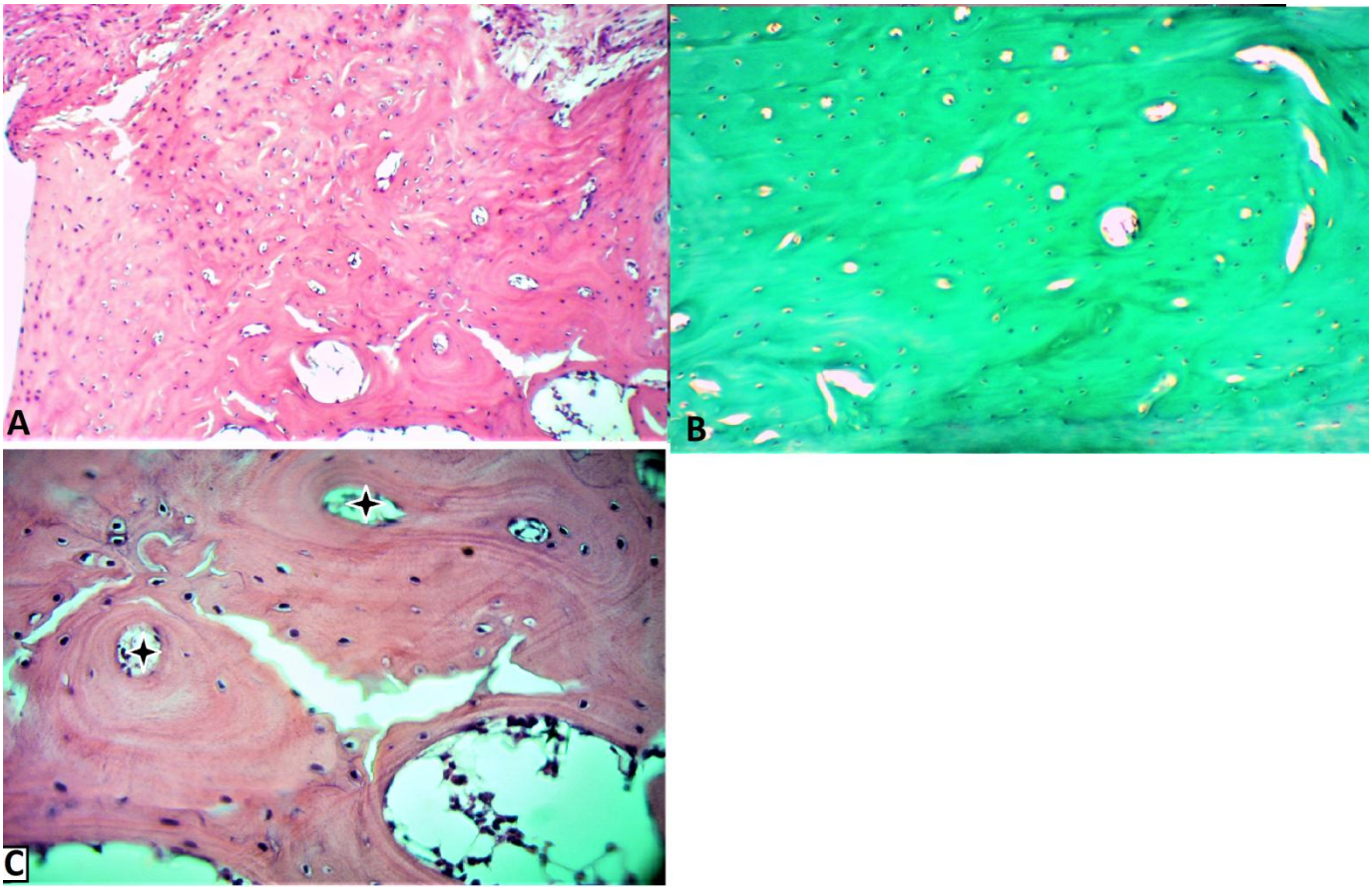
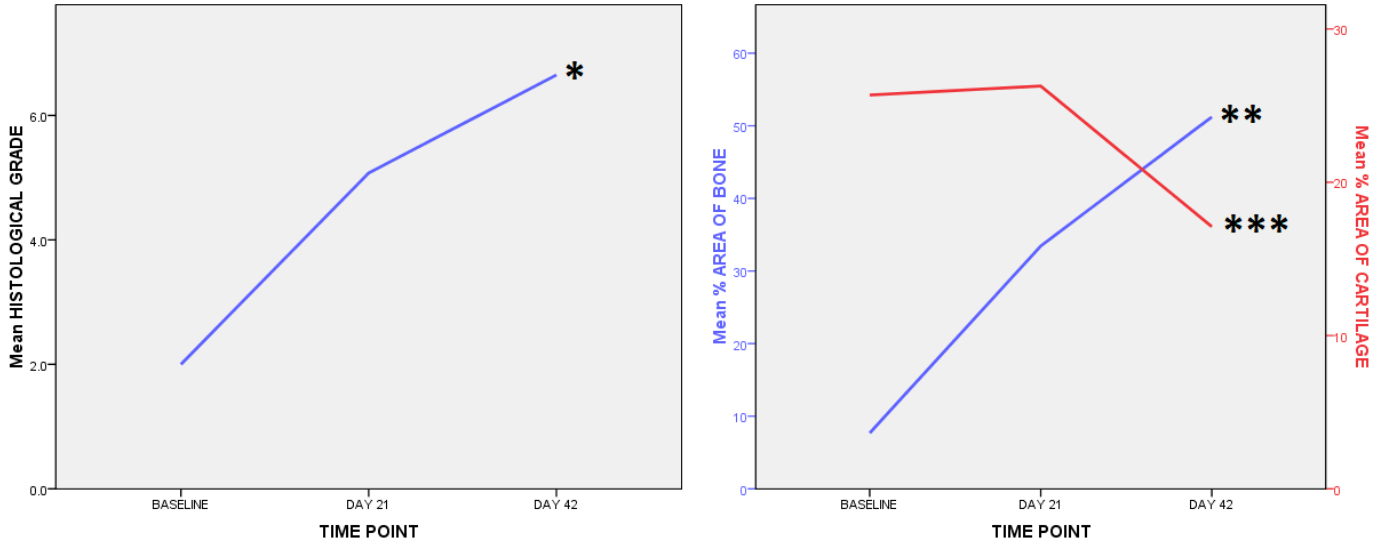


Figure 8 A-C. Photomicrograph of the fracture callus of a rat tibia on day 42 in rats that received diclofenac

4.1.2.2 Histomorphometry and Stereology of the Temporal Changes of the Rat Tibia Fracture Callus with the use of NSAIDs

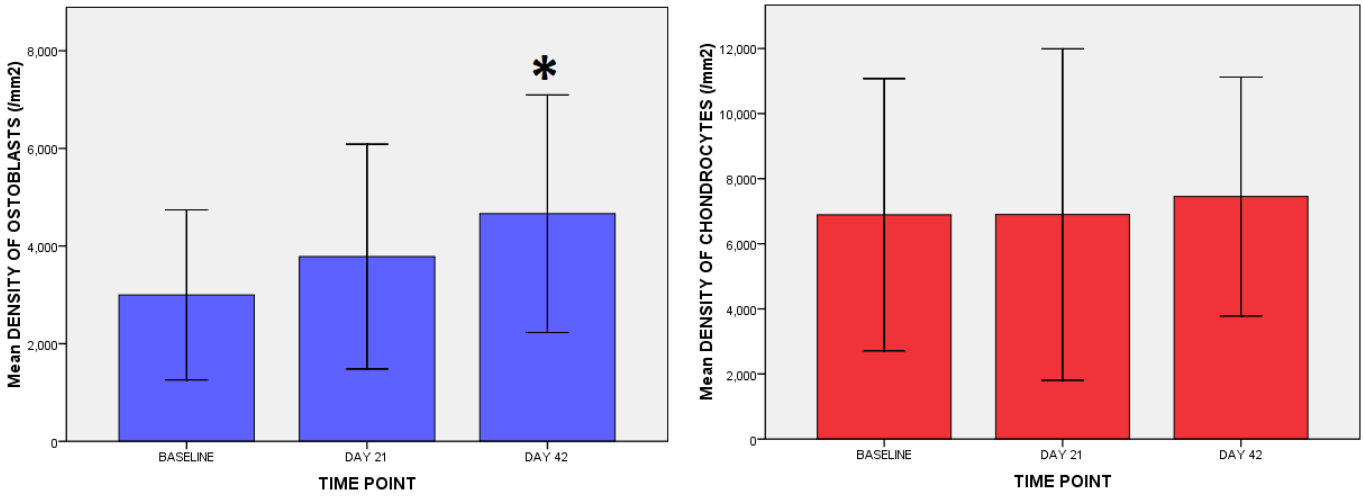
The histological grade increased from the baseline on day 7 to day 42 and this was statistically significant (Figure 9). Post hoc analysis revealed significant differences between days 7 and 21 and days 21 and 42 ($p<0.001$). The proportion of bone in the fracture callus also increased from day 7 to day 42 (Figure 9). Post hoc analysis revealed significant differences between days 7 and 21 and days 21 and 42 ($p<0.001$). The proportion of cartilage is also reduced in this period and this was statistically significant (Figure 9). Post hoc analysis revealed differences between day 42 and baseline ($p=0.035$), and day 21 ($p=0.001$).



*p<0.001 **p<0.001 ***p=0.001 using one-way ANOVA

Figure 9. Histomorphometric parameters of the healing fracture callus

The osteoblast density increases significantly in the period between the baseline on day 7, through days 21 and 42 (Figure 10). Post hoc analysis revealed differences between day 42 and baseline (p=0.005), and day 21 (p=0.003). The chondrocyte density was similar from the baseline and day 21 and increased from day 21 to day 42. These changes were not statistically significant (Figure 10).



*p<0.001 using one-way ANOVA

Figure 10. Stereological parameters of the healing fracture callus.

4.2 STRUCTURE OF THE RAT TIBIA FRACTURE CALLUS COMPARING DICLOFENAC AND CELECOXIB

The fracture callus exhibits differences when the rats that received diclofenac and celecoxib are compared. The celecoxib group shows slower conversion of mesenchymal fibrous tissue to bone with the persistence of cartilage in the later stages of fracture healing. The celecoxib group was also noted to have lower histological grades and proportion of bone. The cell densities were similar between the groups studied.

4.2.1 Histomorphology of the Rat Tibia Fracture Callus Comparing Diclofenac and Celecoxib

On day 21 the animals that received diclofenac demonstrate a highly cellular fracture callus that mainly has cartilage and bone. The matrix in the areas of bone stains deeply with numerous vascular channels and multiple small rounded darkly staining cells. The areas of cartilage have a clear matrix and larger cells with prominent nuclei (Figure 11A). The areas with bone demonstrate a vascular matrix with islands of immature bone. The oval-shaped channels are separated from each other by islands of immature bone. The cells are in lacunae and have darkly staining nuclei. There are also several multinucleated osteoclasts on the surfaces of the newly formed bone (Figure 11B). The areas of cartilage are avascular with closely packed cells with a scanty matrix. The cells are large and rounded with prominent nuclei (Figure 11C).

Figure 11 A-C. Photomicrograph of the tibia fracture callus on day 21 in the rats that received diclofenac early

A: Fracture callus showing the fractured end (white diamond), bone and cartilage areas [Haematoxylin and Eosin stain, X100].

B: Higher magnification of the section in Figure 11A showing the areas with bone. Notice the small rounded cells in lacunae surrounding a vascular channel (star) and osteoclasts (down-facing arrow) [Haematoxylin and Eosin stain, X400].

C: Higher magnification of the section in Figure 11A showing cartilage areas with large rounded cells in lacunae in an amorphous matrix. Notice the large prominent nucleus (right-facing arrow) [Haematoxylin and Eosin stain, X400].

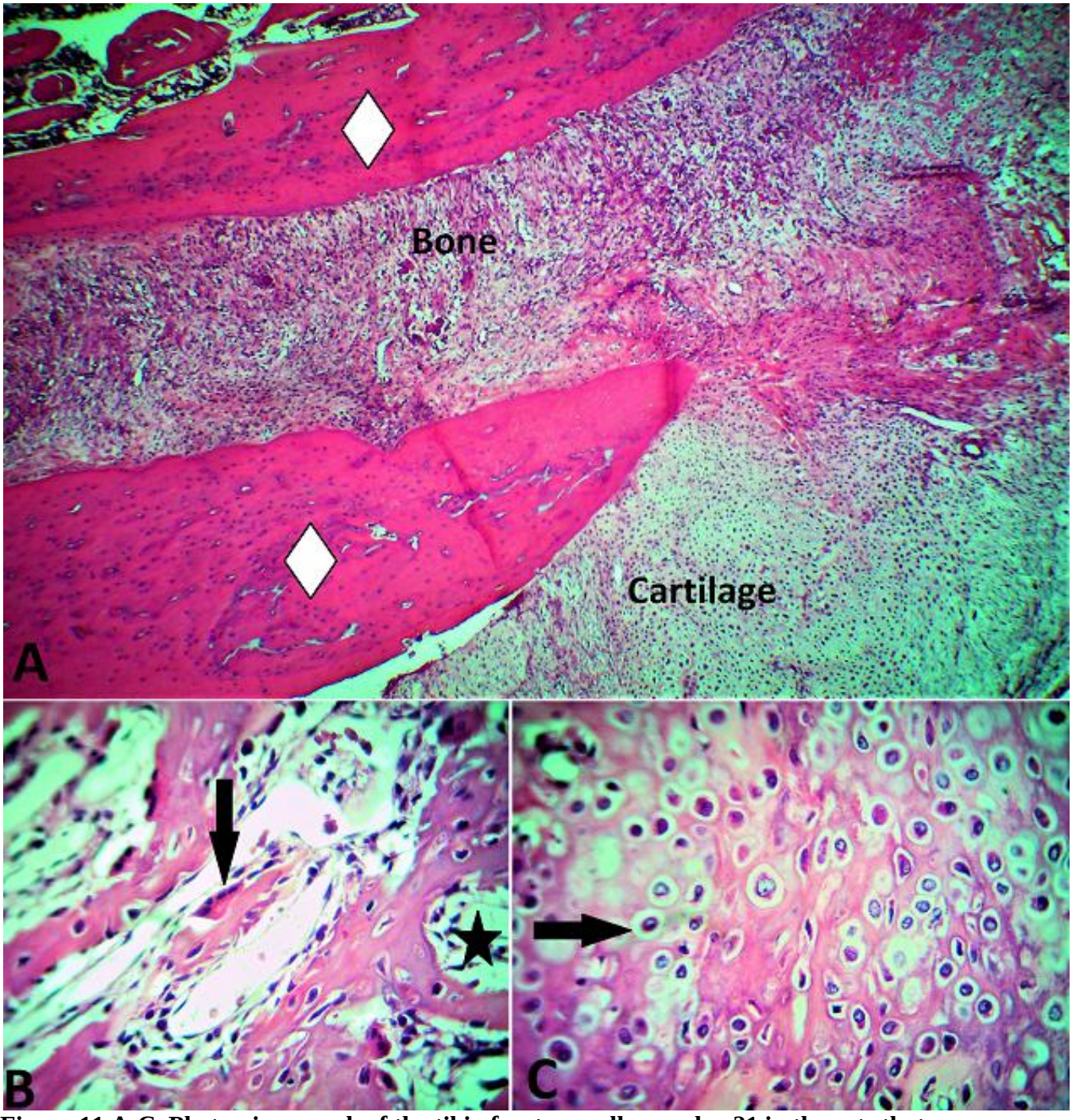


Figure 11 A-C. Photomicrograph of the tibia fracture callus on day 21 in the rats that received diclofenac early [Haematoxylin and Eosin stain].

By day 42, the healing tibia fracture callus is comprised mainly of bone with scanty cartilage, especially in the areas near the rugged fractured end of the tibia. There are also areas of cartilage scattered in the healing callus mixed with immature bone with numerous irregularly shaped vascular channels. While the matrix stains well, the cells are darkly staining with the osteoblasts being smaller while the chondrocytes are larger (Figure 12A). The osteoblasts are found lining the vascular channels and have an irregularly oriented matrix between them. While some cells are flattened, many are rounded with prominent dark staining nuclei. Osteoclasts are rare, indicating the tissue is in the bone-laying stages of healing (Figure 12B). The areas with cartilage have an avascular, featureless matrix. The cells are in isogenous groups with little matrix between them. The cells are large, found in lacunae, and have diffuse nuclear material. Some cells are in different stages of cell division, indicating a tissue that is rapidly proliferating (Figure 12B and C).

Figure 12 A-C. Photomicrograph of the tibia fracture callus on day 42 in the rats that received diclofenac early

A: Fracture callus showing the fractured end (white diamond) with mainly bone [Haematoxylin and Eosin stain, X100].

B: Higher magnification of the section in Figure 12A showing areas of bone. Notice the small rounded cells in lacunae surrounding a vascular channel (star) and some chondrocytes (right-facing arrow) [Haematoxylin and Eosin stain, X400].

C: Higher magnification of the section in Figure 12A showing bone and cartilage interface areas. Notice the diffuse nuclear material with vascularisation [Haematoxylin and Eosin stain, X400].

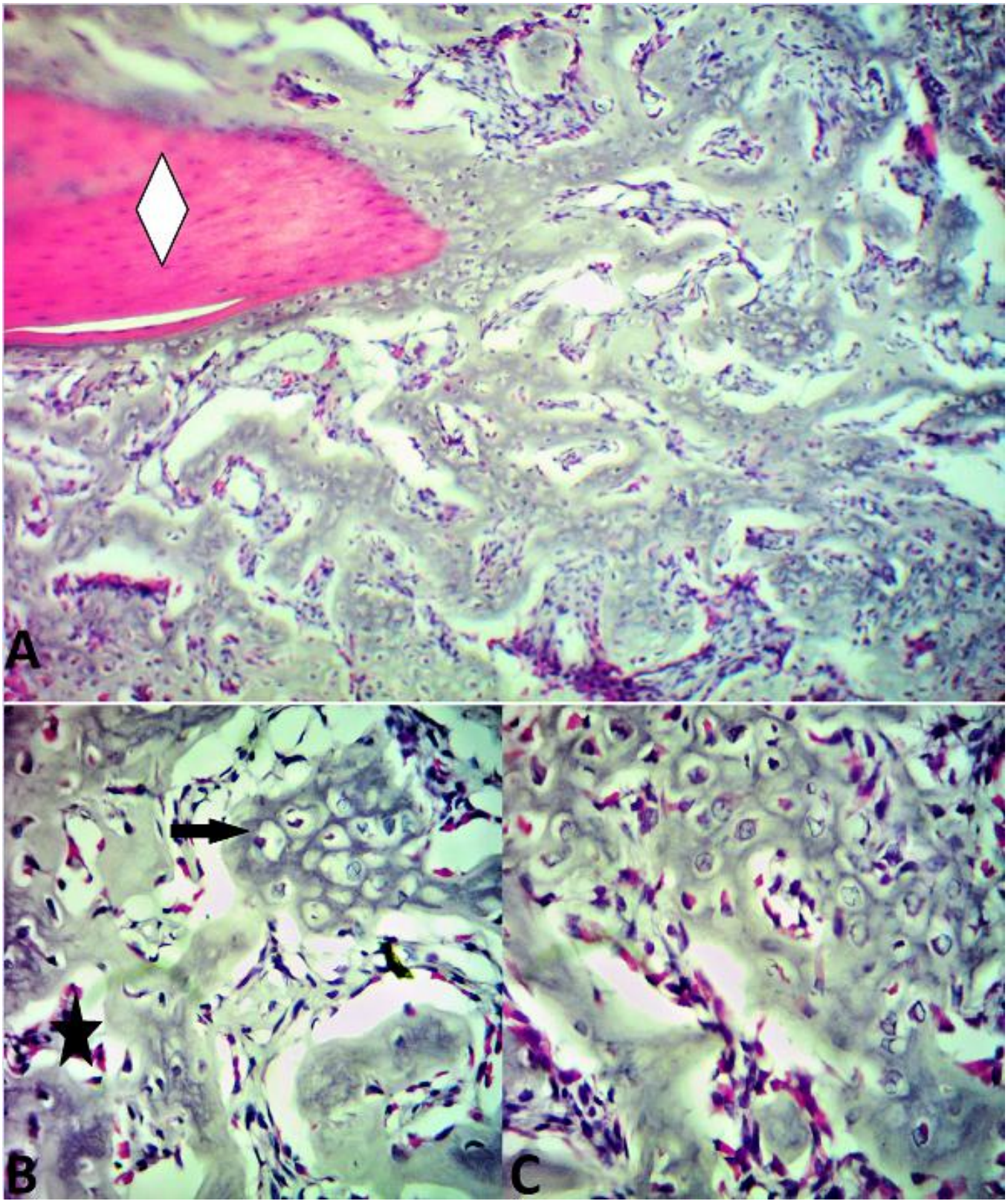


Figure 12 A-C. Photomicrograph of the tibia fracture callus on day 42 in the rats that received diclofenac early [Haematoxylin and Eosin stain].

The tibiae of animals that got celecoxib also show a highly cellular callus on day 21 near the fractured end of the bone. The rest of the callus is also highly cellular with little intercellular matrix noted. The callus is relatively immature with a notable presence of mesenchymal fibrous tissue and cartilage with no bone. The matrix is relatively avascular with only a few vascular channels noted and stains better in the areas of the mesenchymal fibrous tissue (Figure 13A). The areas with cartilage have numerous cells with a little matrix that is plain and stains poorly. The chondrocytes are in isogenous groups and show evidence of cell division. The cells are found in lacunae, are mainly rounded in shape, and have prominent nuclei (Figure 13B). The mesenchymal fibrous tissue has a fibrous matrix with irregularly arranged fibers. The numerous spindle-shaped cells have dark staining nuclei while a few of the cells are rounded (Figure 13C).

Figure 13 A-C. Photomicrograph of the tibia fracture callus on day 21 in the rats that received celecoxib early

A: Fracture callus showing the fractured end (white diamond) with areas of bone and cartilage [Haematoxylin and Eosin stain, X100].

B: Higher magnification of the section in Figure 13A showing cartilage. Notice the recently divided cells (right-facing arrow) [Haematoxylin and Eosin stain, X400].

C: Higher magnification of the section in Figure 13A showing mesenchymal fibrous tissue. Notice the spindle-shaped cells in a fibrous matrix [Haematoxylin and Eosin stain, X400].

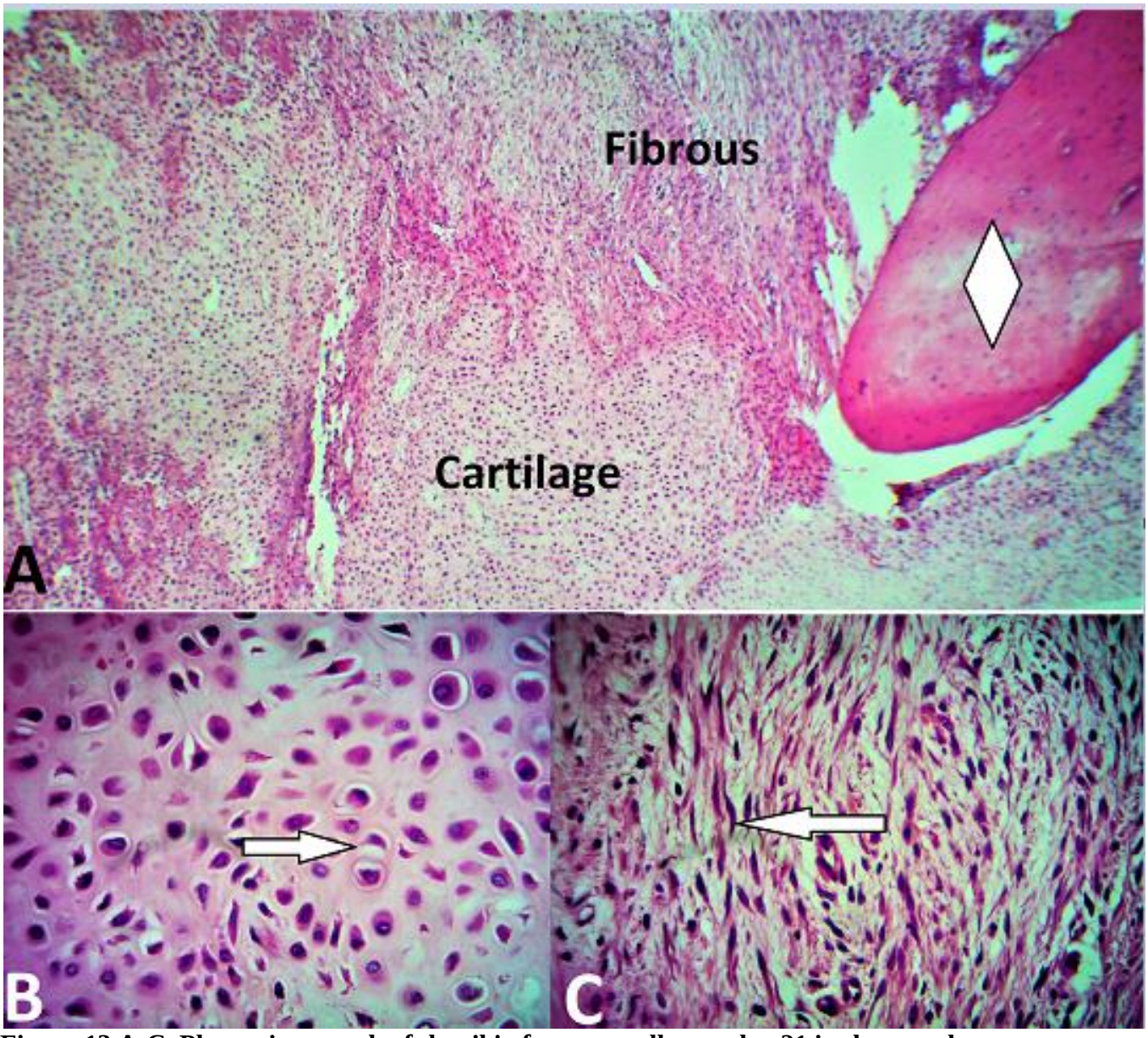


Figure 13 A-C. Photomicrograph of the tibia fracture callus on day 21 in the rats that received celecoxib early [Haematoxylin and Eosin stain].

On day 42 the fracture callus is mainly composed of cartilage with a little bone. While the matrix of the areas of cartilage has a plain matrix, the areas of bone have a matrix that takes up stain well (Figure 14A). The bone is vascularized and has numerous small darkly staining osteoblasts and several multinucleated osteoclasts (Figure 14B). The areas of cartilage have numerous large cells in lacunae with scanty matrix between them. The cells are arranged in isogenous groups with some showing evidence of cell division (Figure 14C). The areas with mesenchymal fibrous tissue have a fibrous matrix with haphazardly oriented fibers and few vascular channels. The numerous cells are scattered throughout the tissue with the majority being spindle-shaped and darkly staining (Figure 14D)

Figure 14 A-D. Photomicrograph of the tibia fracture callus on day 42 in the rats that received celecoxib early

A: Fracture callus showing areas of bone and cartilage [Haematoxylin and Eosin stain, X100].

B: Higher magnification of the section in Figure 14A showing areas of bone. Notice the multinucleated osteoclasts (down-facing arrow) [Haematoxylin and Eosin stain, X400].

C: Higher magnification of the section in Figure 14A showing cartilage areas. Notice the spindle-shaped cells in isogenous groups (right-facing arrow) [Haematoxylin and Eosin stain, X400].

D: Higher magnification of the section in Figure 14A showing mesenchymal fibrous tissue. Notice the sparsely distributed cells in a fibrous matrix [Haematoxylin and Eosin stain, X400].

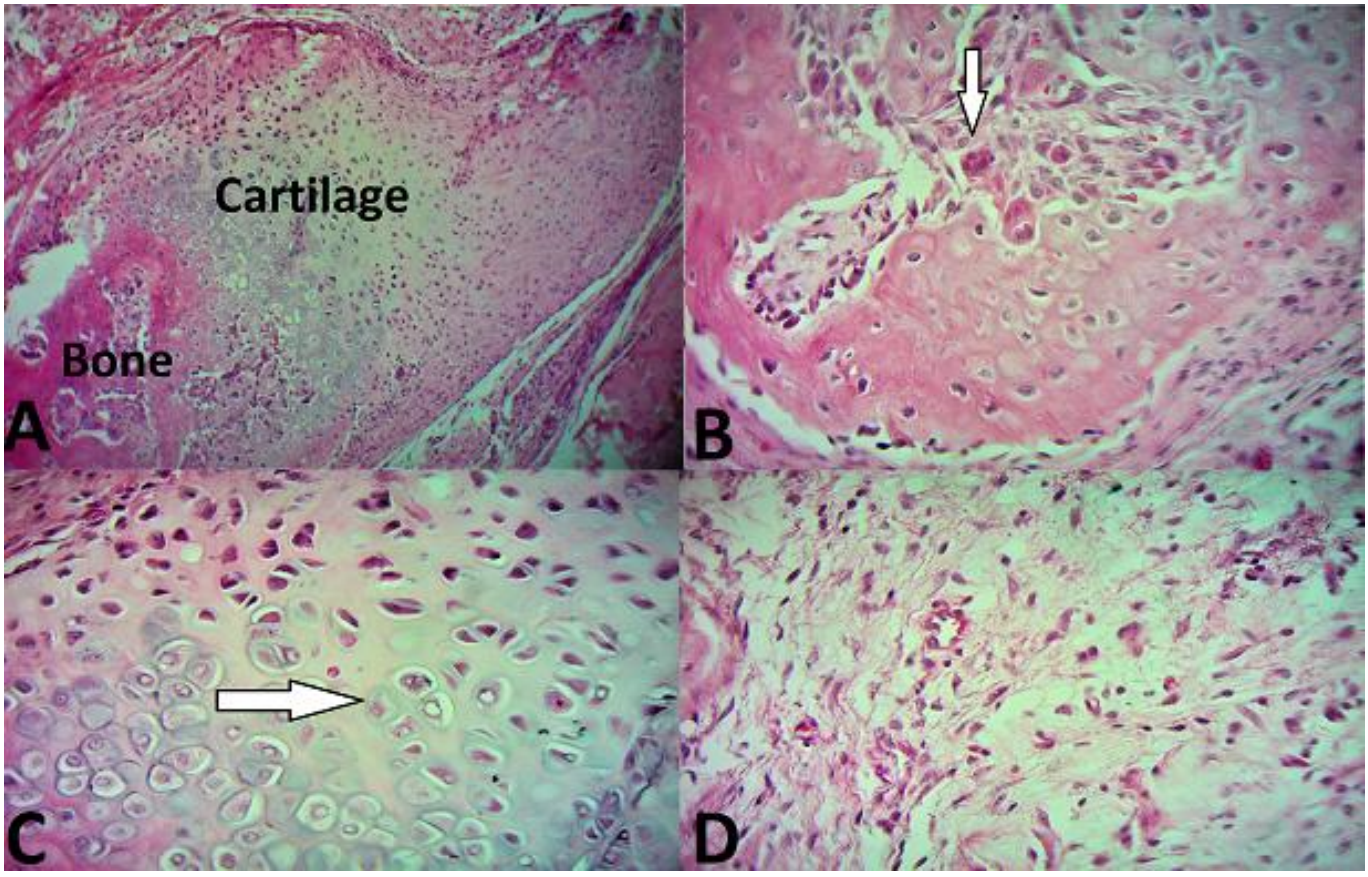


Figure 14 A-D. Photomicrograph of the tibia fracture callus of the rats that received celecoxib early on day 42 [Haematoxylin and Eosin stain].

4.2.2 Histomorphometry of the Rat Tibia Fracture Callus Comparing Diclofenac and Celecoxib

The histological grade of the diclofenac group is higher than that of the celecoxib group, while the proportion of cartilage is higher in the celecoxib group on day 21. These differences are not statistically significant. The proportion of bone is higher in the diclofenac group and this is statistically significant. On day 42 the group with diclofenac has a statistically higher histological grade and a higher proportion of bone. The diclofenac group also has a lower proportion of cartilage, though this is not statistically significant (Table 1).

Table 1. Histomorphometric parameters comparing diclofenac and celecoxib

	Histological Grade value(SD)		Proportion of Bone %(SD)		Proportion of Cartilage %(SD)	
	Diclofenac	Celecoxib	Diclofenac	Celecoxib	Diclofenac	Celecoxib
Day 21	5.40 (1.39)	4.75 (1.21)	37.08 (7.62)	29.80 (11.17)*	24.67 (11.90)	27.88 (16.06)
Day 42	7.20 (0.69)	6.10 (1.45)**	56.71 (7.41)	46.45 (10.85)***	16.85 (5.05)	17.17 (7.89)

*p=0.021 **p=0.004 ***p=0.001 using the student T-test

4.2.3 Stereology of the Rat Tibia Fracture Callus Comparing Diclofenac and Celecoxib

The diclofenac group has a higher osteoblast density on day 21 but by day 42 the celecoxib group has the higher density. The diclofenac group has a higher chondrocyte density on both days 21 and 42 (Figure 15). These differences are not statistically significant.

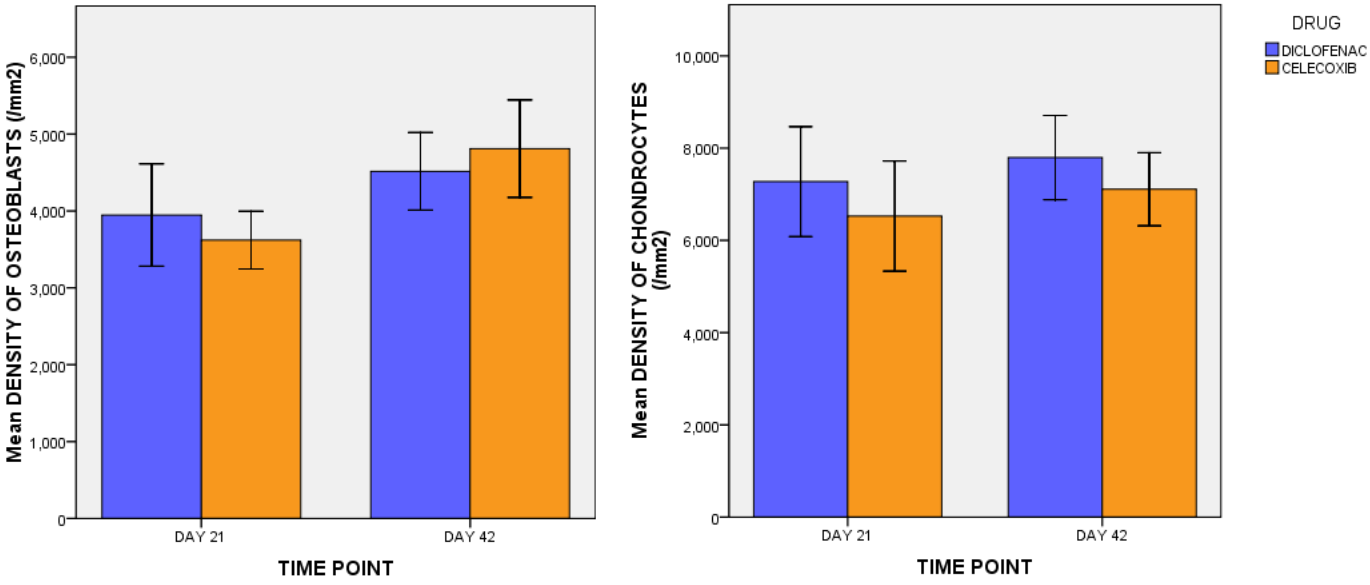


Figure 15. Graph of stereological parameters comparing diclofenac and celecoxib

4.3 STRUCTURE OF THE RAT TIBIA FRACTURE CALLUS COMPARING DIFFERENT TIMINGS OF NSAIDS

The fracture callus displays differences when the rats that received NSAIDs early and late are compared with the former showing persistence of cartilage and a lower proportion of bone. The late group also has a higher histological grade and proportion of bone with a lower proportion of cartilage. While the early group has higher osteoblast densities, the chondrocyte densities are similar between the two groups.

4.3.1 Histomorphology of the Rat Tibia Fracture Callus Comparing Different Timings of NSAIDs

The animals that received the study medication early showed an almost equal proportion of immature bone, cartilage, and mesenchymal fibrous tissue on day 21 (Figure 16A). While the cartilage and fibrous areas are avascular, the bone is characterized by large vascular spaces. The bone has small, rounded, darkly staining cells. Some of the cells and matrix show a tendency to a concentric arrangement around vascular channels (Figure 16B). The cartilage has large rounded cells in lacunae. The cells are closely packed and arranged in columns with very little extracellular matrix (Figure 16C).

Figure 16 A-C. Photomicrograph of the tibia fracture callus on day 21 in the rats that received diclofenac early

A: Fracture callus showing the fractured end (white diamond) with equal areas of bone, cartilage, and mesenchymal fibrous tissue [Haematoxylin and Eosin stain, X100].

B: Higher magnification of the section in Figure 16A showing areas with bone tissue. Notice the small rounded cells in lacunae surrounding a vascular channel (star) [Haematoxylin and Eosin stain, X400].

C: Higher magnification of the section in Figure 16A showing cartilage areas. Notice the large cells in lacunae and their prominent nuclei (right-facing arrow) [Haematoxylin and Eosin stain, X400].

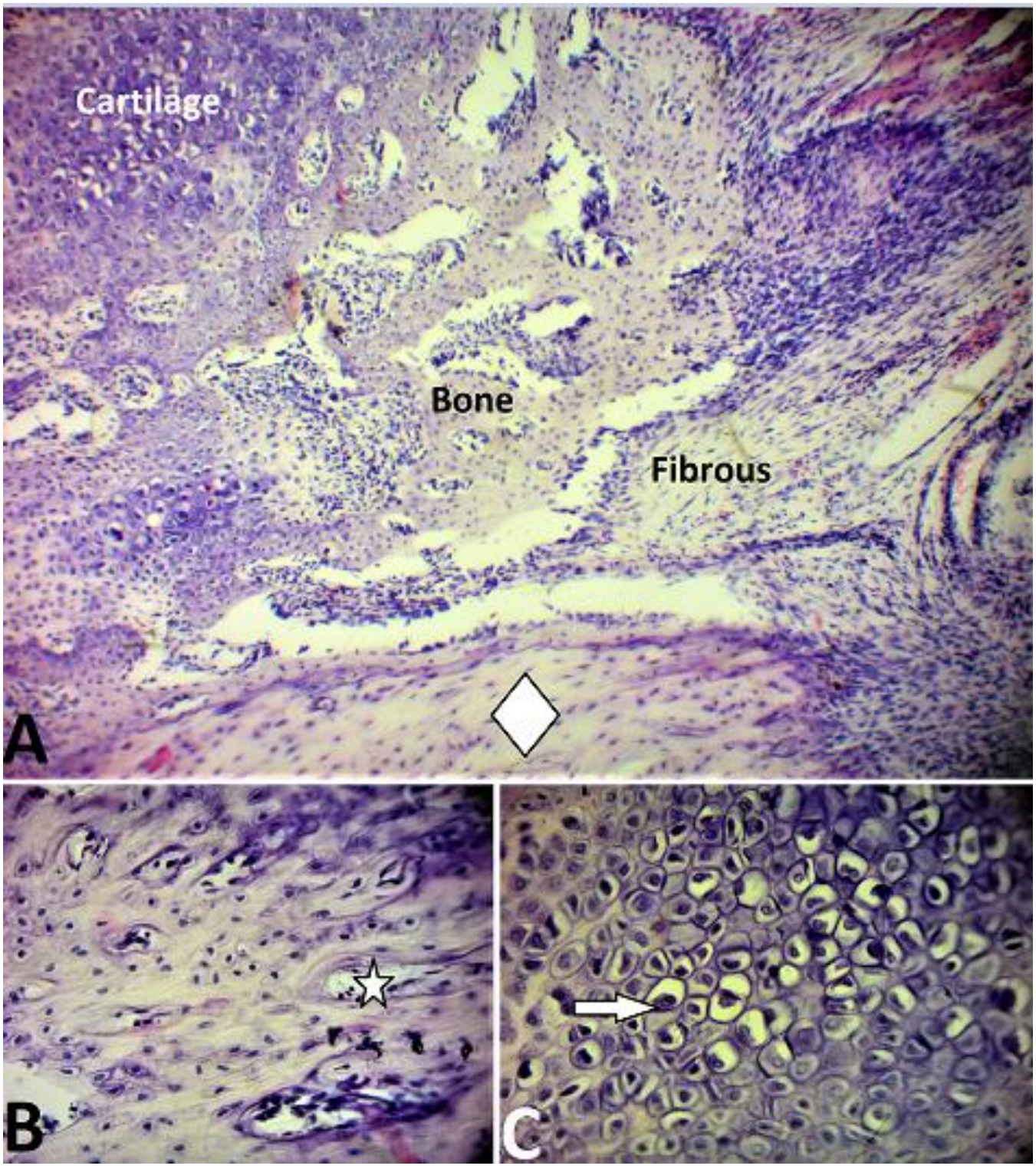


Figure 16 A-C. Photomicrograph of the tibia fracture callus on day 21 in the rats that received diclofenac early [Haematoxylin and Eosin stain].

By day 42 the fracture callus is mainly composed of cartilage and immature bone. The areas of cartilage have an avascular featureless matrix compared to the areas of bone which have a deeply staining vascularised matrix (Figure 17A). The areas of bone show a transition from cartilage with larger chondroid-looking cells interspersed with small darkly staining osteoblasts. Multinucleated osteoclasts are also prominent adjacent to the vascular channels with darkly staining nuclei with a brightly staining cytoplasm (Figure 17B). The cartilage areas have large chondrocytes in isogenous groups with scanty featureless matrix between them. The cells, found in lacunae, are undergoing rapid cell division evidenced by cells in different stages of the process (Figure 17C).

Figure 17 A-C. Photomicrograph of the tibia fracture callus on day 42 in the rats that received diclofenac early

A: Fracture callus showing the fractured end (white diamond) with immature bone and cartilage [Haematoxylin and Eosin stain, X100].

B: Higher magnification of the section in Figure 17A showing areas of bone. Notice the small rounded cells in lacunae surrounding a vascular channel (star) and the multinucleated osteoclasts (down-facing arrow) [Haematoxylin and Eosin stain, X400].

C: Higher magnification of the section in Figure 17A showing cartilage. Notice the large cells in lacunae and a cell that is about to undergo cytokinesis (right-facing arrow) [Haematoxylin and Eosin stain, X400].

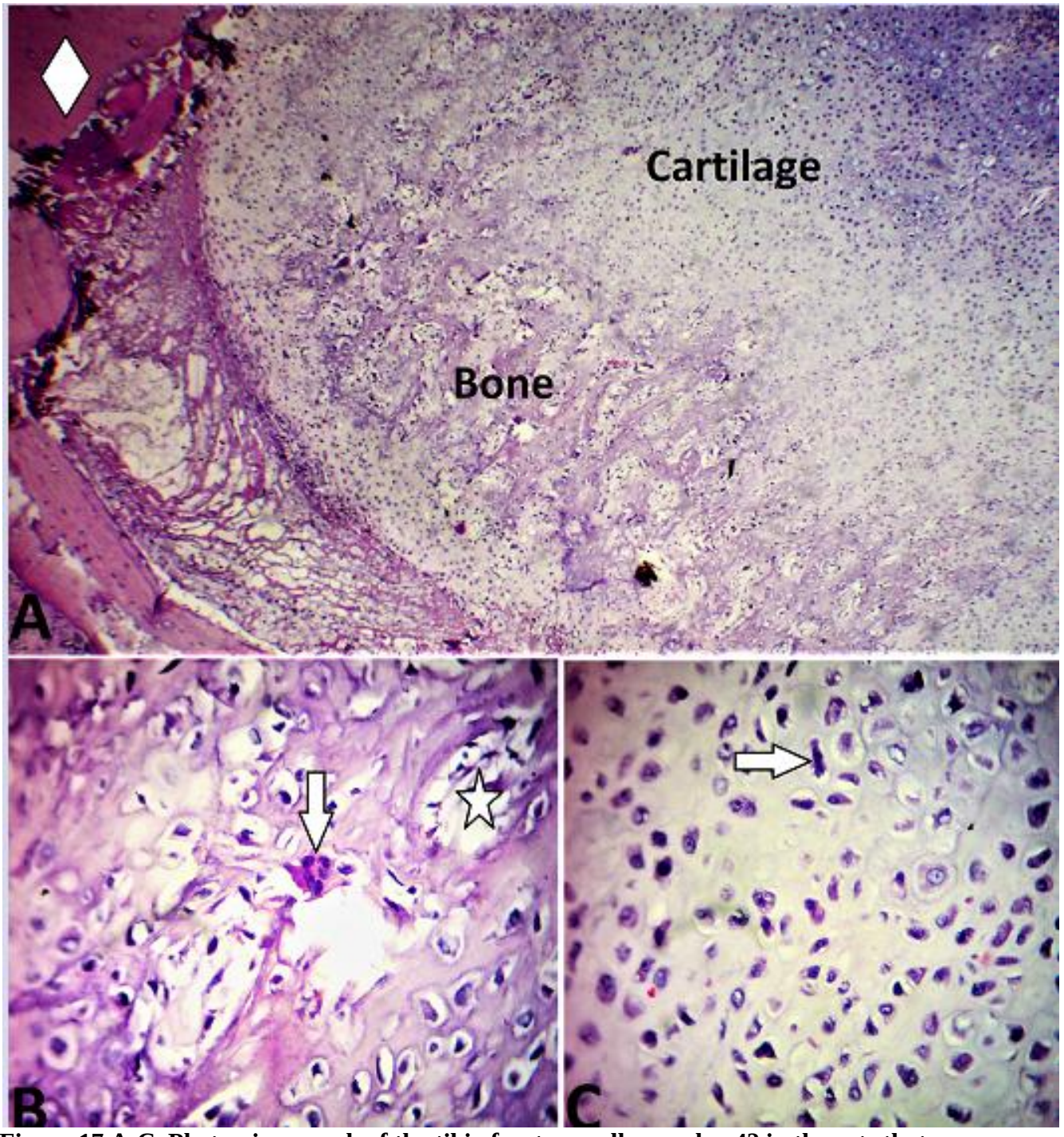


Figure 17 A-C. Photomicrograph of the tibia fracture callus on day 42 in the rats that received diclofenac early [Haematoxylin and Eosin stain].

The fracture callus of the animals in the late group is composed mainly of cartilage and mesenchymal fibrous tissue on day 21. While the chondroid matrix is glassy and doesn't take up stain well, the mesenchymal matrix is fibrous (Figure 18A). The areas of bone also have vascularized cartilage which is transforming into bone. The cells are large with a diffuse nucleus. Smaller, darker staining osteoblasts are found closer to the vessels and no osteoclasts are identified (Figure 18B). The cartilage displays cells in isogenous groups with a relatively scanty matrix. The cells are somewhat smaller and less rounded and most are found in lacunae (Figure 18C). The areas with mesenchymal fibrous tissue show closely packed spindle-shaped cells adjacent to rounded chondroid cells indicating mesenchymal to chondroid transformation (Figure 18D).

Figure 18 A-D. Photomicrograph of the tibia fracture callus on day 21 in the rats that received diclofenac late

A: Fracture callus showing the fractured end (white diamond) with immature bone, cartilage, and mesenchymal fibrous tissue [Haematoxylin and Eosin stain, X100].

B: Higher magnification of the section in Figure 18A showing vascularised cartilage. Notice the large cells with a large nucleus and a vascular channel (star) [Haematoxylin and Eosin stain, X400].

C: Higher magnification of the section in Figure 18A showing cartilage areas. Notice the large cells in lacunae in isogenous groups (right-facing arrow) [Haematoxylin and Eosin stain, X400].

D: Higher magnification of the section in Figure 18A showing the interface areas. Notice the transition from the mesenchymal fibrous tissue to cartilage and then vascularised cartilage [Haematoxylin and Eosin stain, X400].

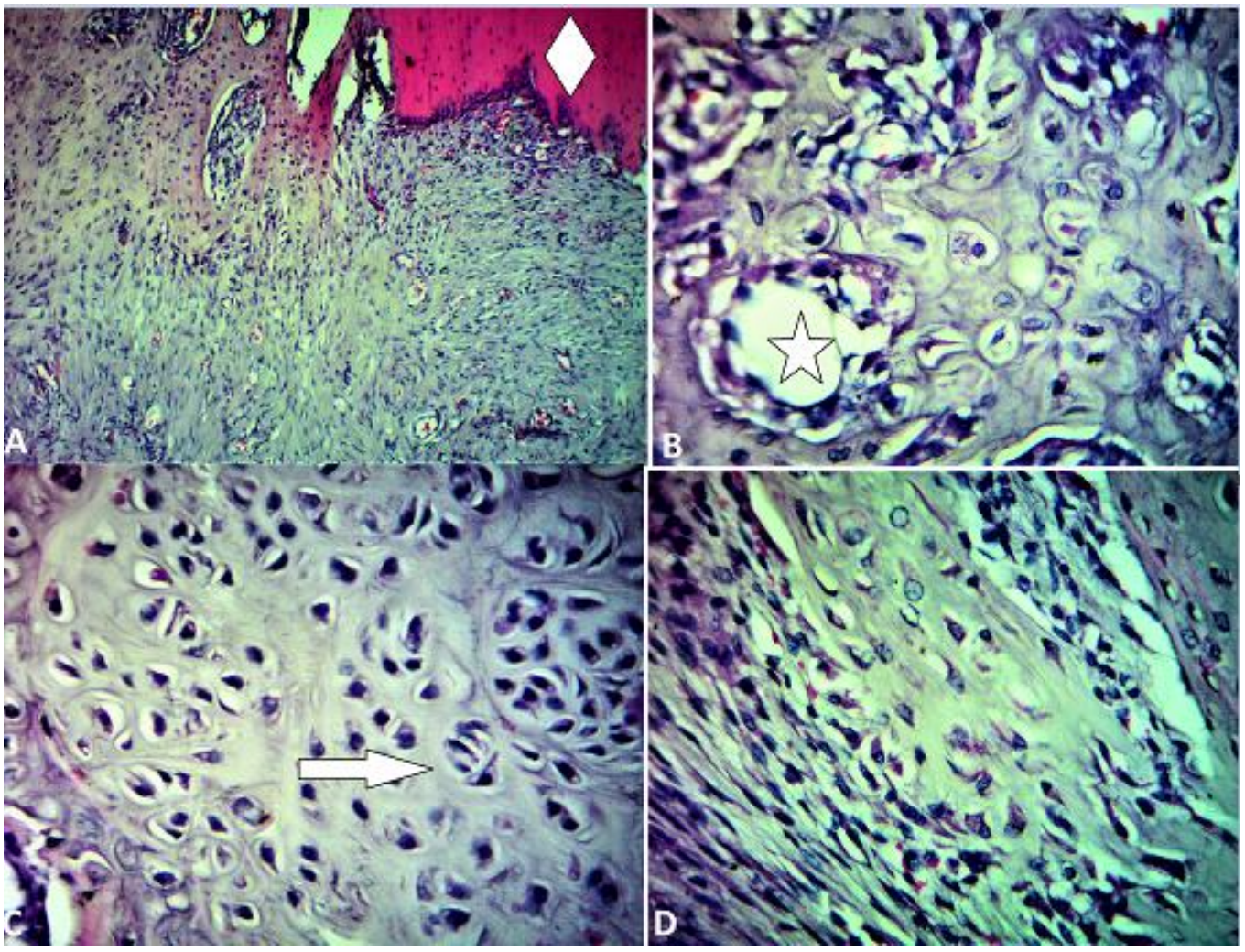


Figure 18 A-D. Photomicrograph of the tibia fracture callus on day 21 in the rats that received diclofenac late [Haematoxylin and Eosin stain].

By day 42 the fracture callus is composed of mainly bone with only a small area of cartilage. While some of the regions with bone show many large vascular channels with immature bone, other areas show a more organized mature matrix (Figure 19A). At higher magnification, there is evidence of the transformation of cartilage to bone. Relatively well-developed Haversian canals are also evident (Figure 19B). The cartilage demonstrates large cells in lacunae within a poorly staining matrix. There is evidence of rapid growth with cells in different stages of cell division (Figure 19C).

Figure 19 A-C. Photomicrograph of the tibia fracture callus on day 42 in the rats that received diclofenac late

A: Fracture callus showing the fractured end (white diamond) with mainly bone with an island of cartilage [Haematoxylin and Eosin stain, X100].

B: Higher magnification of the section in Figure 19A showing areas of bone. Note the small darkly stained cells and the vascular spaces (star). Also note the arrangement of the cells around a Haversian canal (up-facing arrow) [Haematoxylin and Eosin stain, X400].

C: Higher magnification of the section in Figure 19A showing cartilage areas. Notice the large cells in lacunae in isogenous groups (right-facing arrow) [Haematoxylin and Eosin stain, X400].

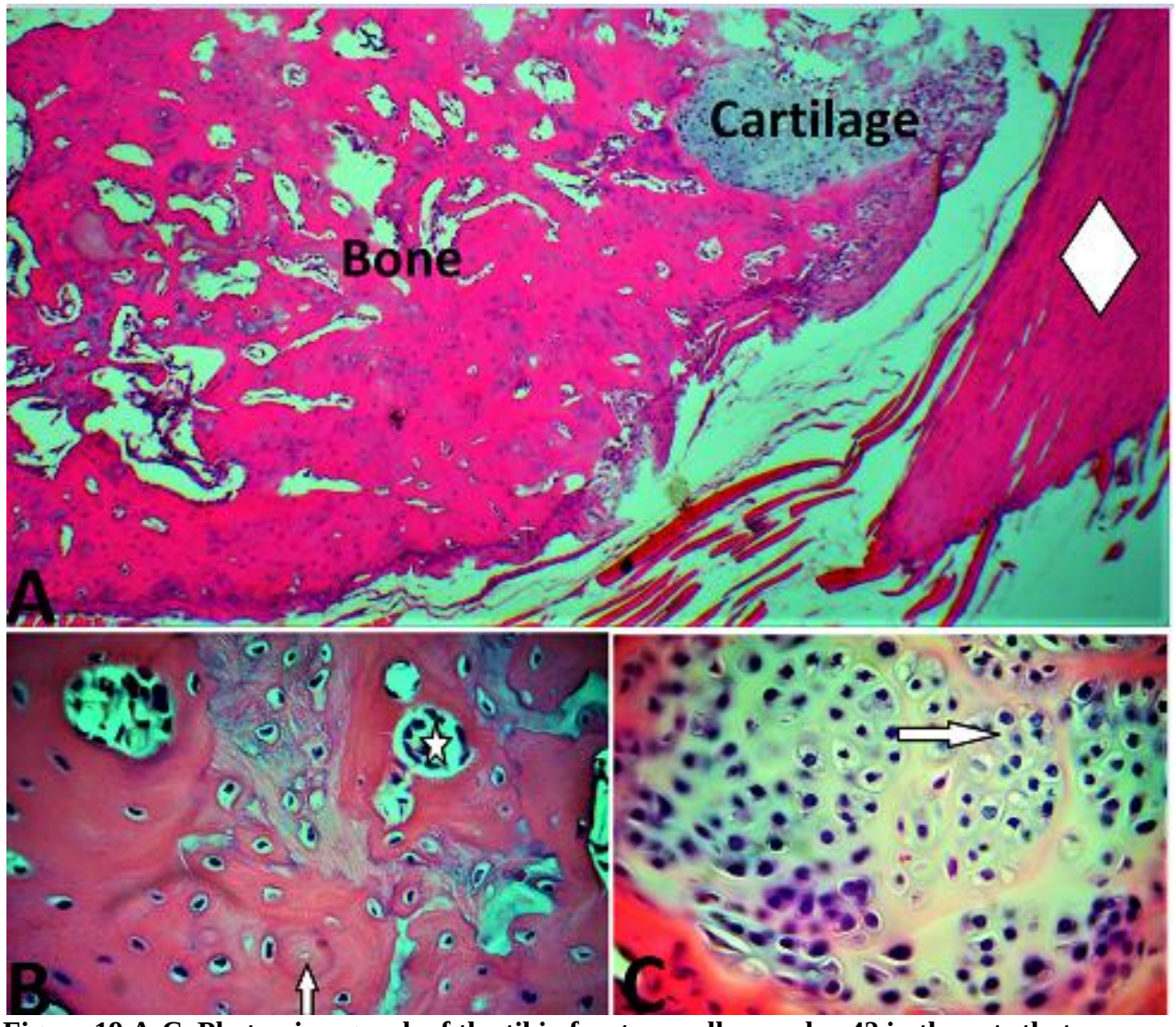


Figure 19 A-C. Photomicrograph of the tibia fracture callus on day 42 in the rats that received diclofenac late [Haematoxylin and Eosin stain].

4.3.2 Histomorphometry of the Rat Tibia Fracture Callus Comparing Different Timings of NSAIDs

On day 21, the animals that received the NSAIDs late have a higher histological grade and a larger proportion of bone and cartilage. These differences are not statistically significant. On day 42 animals that received the medication early have a lower histological grade and a higher proportion of cartilage. These changes are however not statistically significant. The proportion of bone is higher in the late group and this was statistically significant (Table 2).

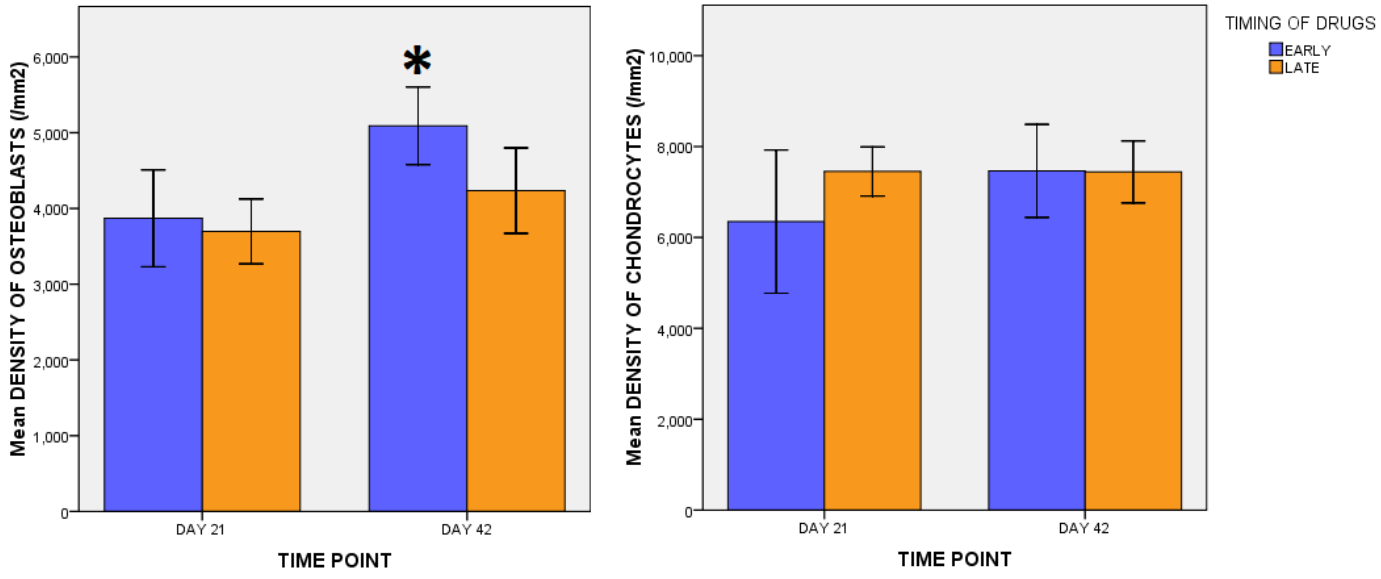
Table 2. Histomorphometric parameters comparing early and late NSAID administration

	Histological Grade value(SD)		Proportion of Bone %(SD)		Proportion of Cartilage %(SD)	
	Early	Late	Early	Late	Early	Late
Day 21	5.00 (1.34)	5.15 (1.35)	31.86 (8.55)	35.03 (11.50)	25.85 (15.33)	26.70 (13.01)
Day 42	6.55 (1.00)	6.75 (1.48)	47.68 (9.32)	55.47 (10.45)*	17.59 (5.98)	16.43 (7.17)

*p=0.017 using the student T-test

4.3.3 Stereology of the Rat Tibia Fracture Callus Comparing Different Timings of NSAIDs

On day 21 the early group has a higher osteoblast density but a lower chondrocyte density. This is however not statistically significant. On day 42, the early group has a higher osteoblast density and this is statistically significant. While the early group has a higher chondrocyte density, this is not statistically significant (Figure 20).



*p=0.024 using the student T-test

Figure 20. Graph of stereological parameters comparing early and late NSAID administration

4.4 STRUCTURE OF THE RAT TIBIA FRACTURE CALLUS WITH AGING

The fracture callus of the rats shows differences depending on the age of the animals with the young animals showing a faster transition to bone formation. The younger animals also have higher proportions of bone and histological grades with lower proportions of cartilage than the older ones. The cell densities do not vary much between the groups studied.

4.4.1 Histomorphology of the Rat Tibia Fracture Callus Comparing Different Age Groups

The fracture callus of the young rats on day 21 is mainly characterized by cartilage, vascularized cartilage, and immature bone. While the matrix in the areas of cartilage is amorphous and stains poorly, the areas of bone have a brightly staining matrix (Figure 21A). The areas of bone are immature with relatively large, darkly staining cells and numerous vascular channels. There are also multinucleated osteoclasts in the borders of the vascular channels (Figure 21B). The areas of cartilage show large cells in lacunae arranged in isogenous groups with very little matrix between them. The cells are in various stages of cell division (Figure 21C).

Figure 21A-C. Photomicrograph of the tibia fracture callus on day 21in the young rats that received diclofenac

A: Fracture callus showing the fractured end (white diamond) with mainly cartilage, vascularized cartilage, and immature bone [Haematoxylin and Eosin stain, X100].

B: Higher magnification of the section in Figure 21A showing areas of bone. Note the transition from vascularized cartilage to the immature bone with small darkly staining cells and the vascular spaces (star). Also note the multinucleated osteoclasts (down-facing arrow) [Haematoxylin and Eosin stain, X400].

C: Higher magnification of the section in Figure 21A showing cartilage areas. Notice the large cells in a lacuna in isogenous groups (right-facing arrow) [Haematoxylin and Eosin stain, X400].

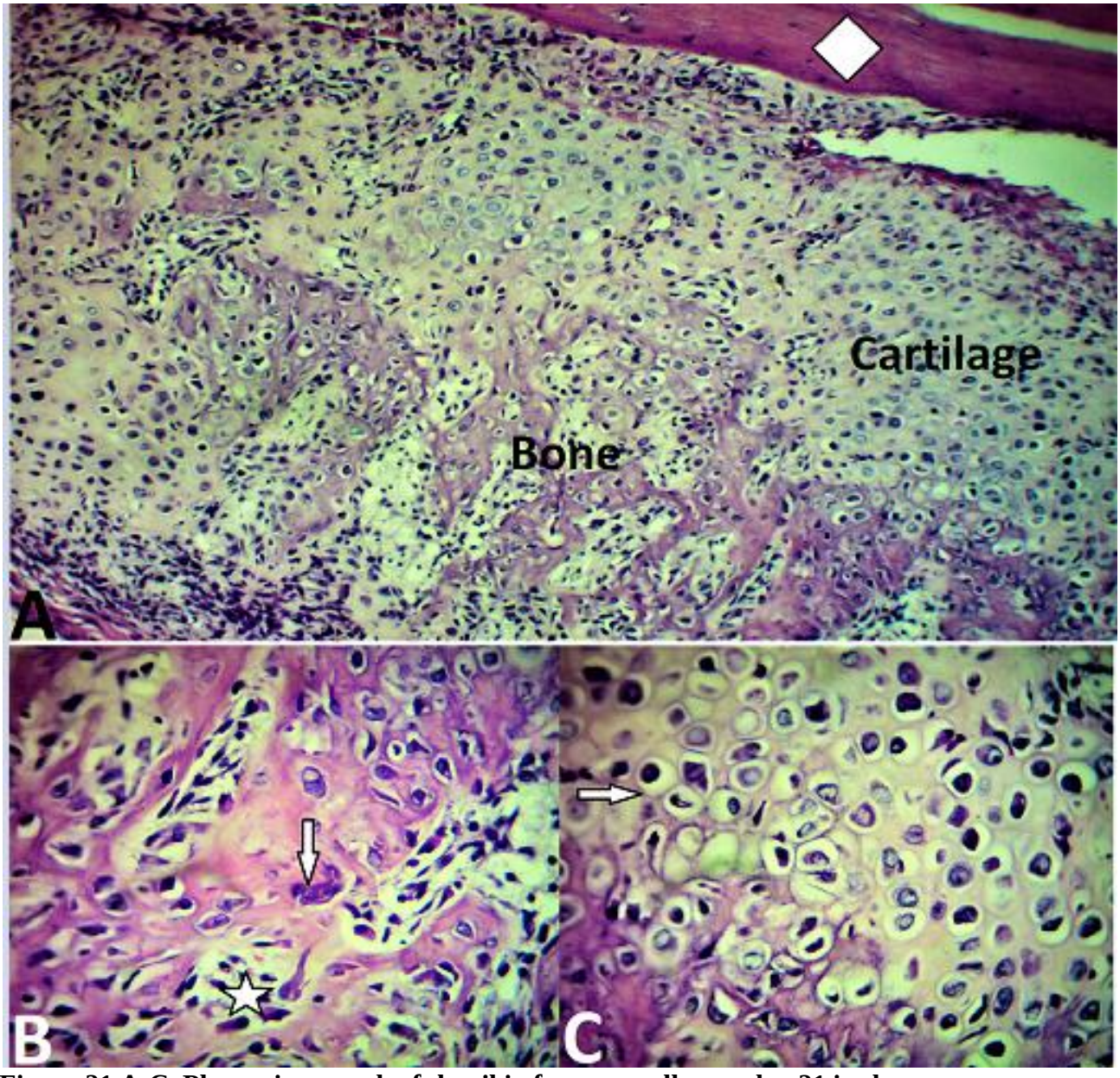


Figure 21 A-C. Photomicrograph of the tibia fracture callus on day 21 in the young rats that received diclofenac [Haematoxylin and Eosin stain].

By day 42 the cartilage in the fracture callus of the young rats is mainly replaced by mature and immature bone leaving a few islands of cartilage. The areas of mature bone have fewer, more rounded vessels compared to the areas of immature bone where the vessels are numerous and irregularly shaped (Figure 22A). At higher magnification, the bone has small, rounded, darkly staining cells with an abundant, regularly oriented matrix. The vascular channels are sparsely distributed and they have multinucleated osteoclasts in the periphery (Figure 22B). The areas of cartilage demonstrate large cells in lacunae arranged in columns. The matrix is sparse and the cells show evidence of cell division (Figure 22C).

Figure 22 A-C. Photomicrograph of the tibia fracture callus on day 42 in the young rats that received diclofenac

A: Fracture callus showing relatively mature bone with a little cartilage [Haematoxylin and Eosin stain, X100].

B: Higher magnification of the section in Figure 22A showing areas of bone. Note the relative paucity of cells and abundance of the intercellular matrix with a few vascular spaces (star) [Haematoxylin and Eosin stain, X400].

C: Higher magnification of the section in Figure 22A showing cartilage areas. Notice the large cells in a lacuna in different stages of cell division (right-facing arrow) [Haematoxylin and Eosin stain, X400].

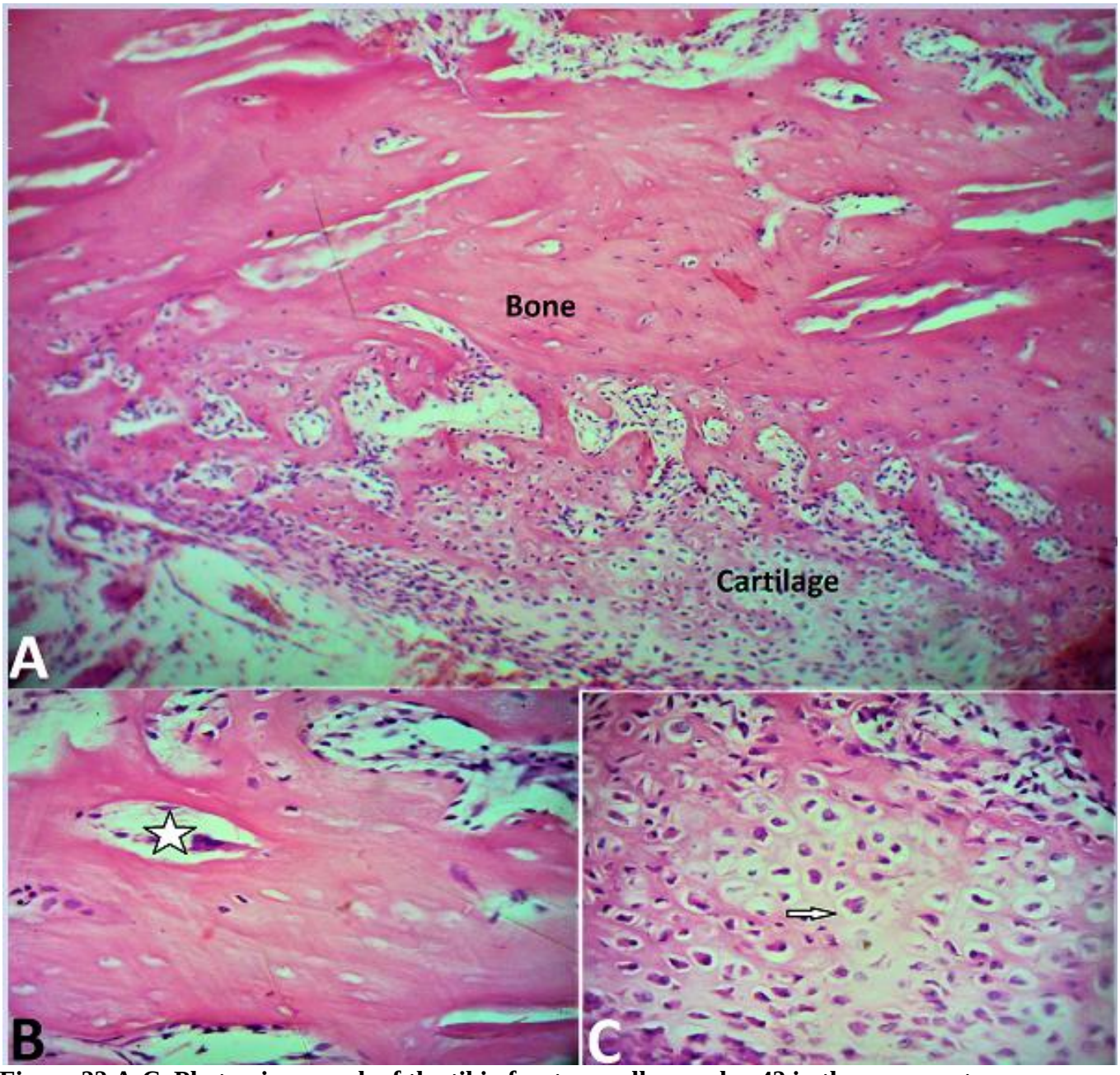


Figure 22 A-C. Photomicrograph of the tibia fracture callus on day 42 in the young rats that received diclofenac [Haematoxylin and Eosin stain].

The fracture callus of the old rats on day 21 exhibits mesenchymal fibrous tissue with scanty cartilage. The mesenchymal fibrous tissue consists of numerous cells in a lightly staining matrix with irregularly oriented fibers (Figure 23A). The mesenchymal cells are spindle-shaped and closely packed in a fibrous matrix that has no discernible vessels (Figure 23B). The cartilage areas have cells in isogenous groups and an avascular matrix. The inter-territorial matrix stains well when compared to the lightly staining territorial matrix (Figure 23C).

Figure 23 A-C. Photomicrograph of the tibia fracture callus on day 21 in the old rats that received diclofenac

A: Fracture callus showing the fractured end (white diamond) and mainly mesenchymal fibrous tissue with little cartilage [Haematoxylin and Eosin stain, X100].

B: Higher magnification of the section in Figure 23A showing mesenchymal fibrous tissue. Notice the spindle-shaped cells in a fibrous matrix [Haematoxylin and Eosin stain, X400]

C: Higher magnification of the section in Figure 23A showing cartilage showing plump rounded cells in lacunae. Notice the cells in isogenous groups and cells in different stages of cell division [Haematoxylin and Eosin stain, X400]

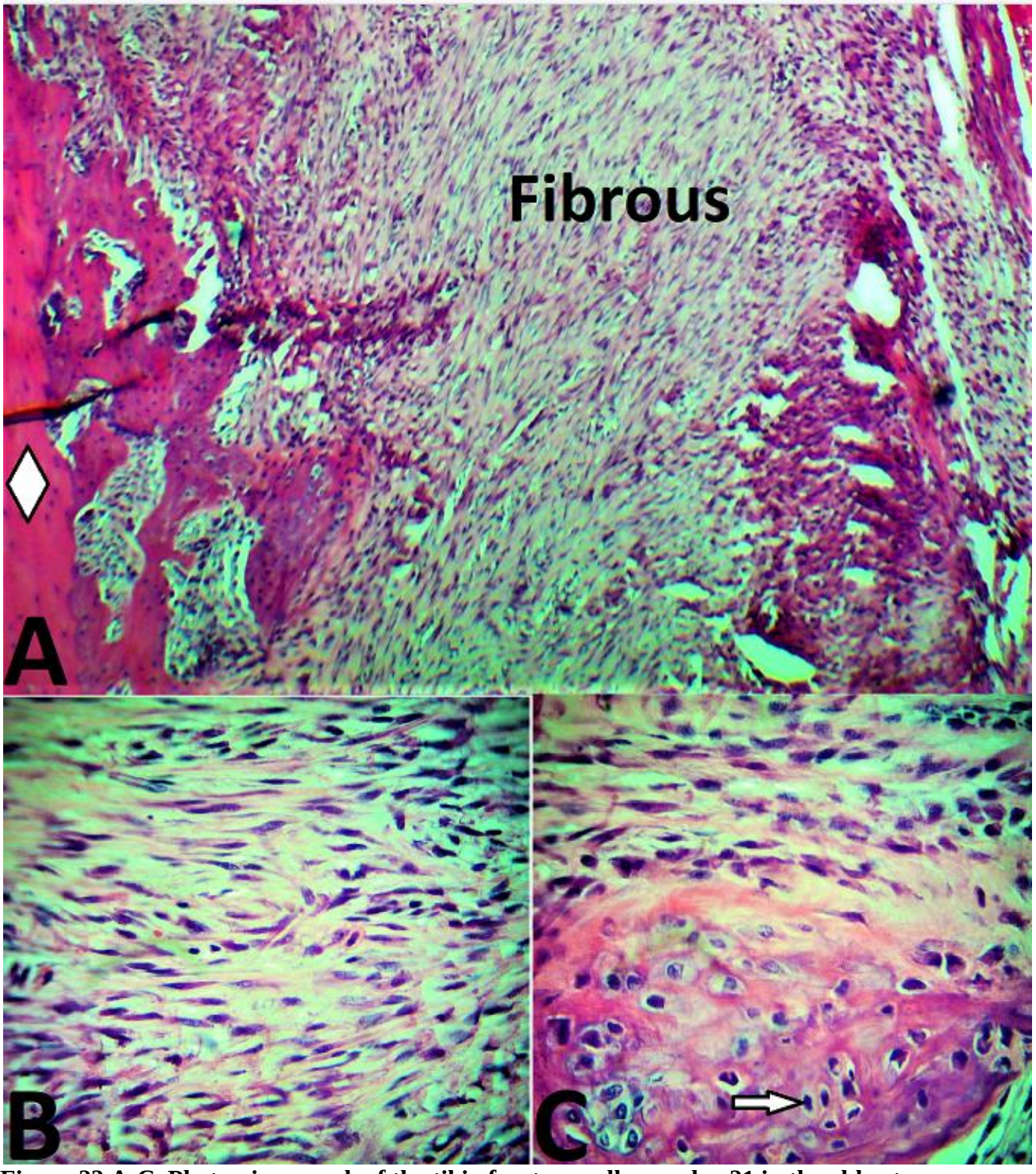


Figure 23 A-C. Photomicrograph of the tibia fracture callus on day 21 in the old rats that received diclofenac [Haematoxylin and Eosin stain].

On day 42 the mesenchymal fibrous tissue in the callus has been replaced by cartilage. There is also a small area of bone. The areas of cartilage and mesenchymal fibrous tissue are avascular when compared to the areas of bone (Figure 24A). The bone is relatively immature with large chondroid-looking cells, a haphazard matrix, and numerous vascular channels. The smaller osteoblasts are closer to the vessels and osteoclasts are rare (Figure 24B). The cartilage has numerous closely packed cells many of which have a diffuse nucleus. There is evidence of mitosis with cells in different stages of division and the matrix is scanty (Figure 24C).

Figure 24 A-D. Photomicrographs of the tibia fracture callus on day 42 in old rats that received diclofenac

A: Fracture callus showing the fractured end (white diamond) and mainly cartilage with a small area of bone. Note the persistence of mesenchymal fibrous tissue [Haematoxylin and Eosin stain, X100].

B: Higher magnification of the section in Figure 24A showing immature bone transitioning from vascularized cartilage with numerous vascular channels (white star) [Haematoxylin and Eosin stain, X400].

C: Higher magnification of the section in Figure 24A showing cartilage showing plump rounded cells in lacunae. Notice the cells in various stages of cell division [Haematoxylin and Eosin stain, X400].

D: Higher magnification of the section in Figure 24A showing mesenchymal fibrous tissue. Notice the spindle-shaped cells with a transition to the plump cells characteristic of cartilage [Haematoxylin and Eosin stain, X400].

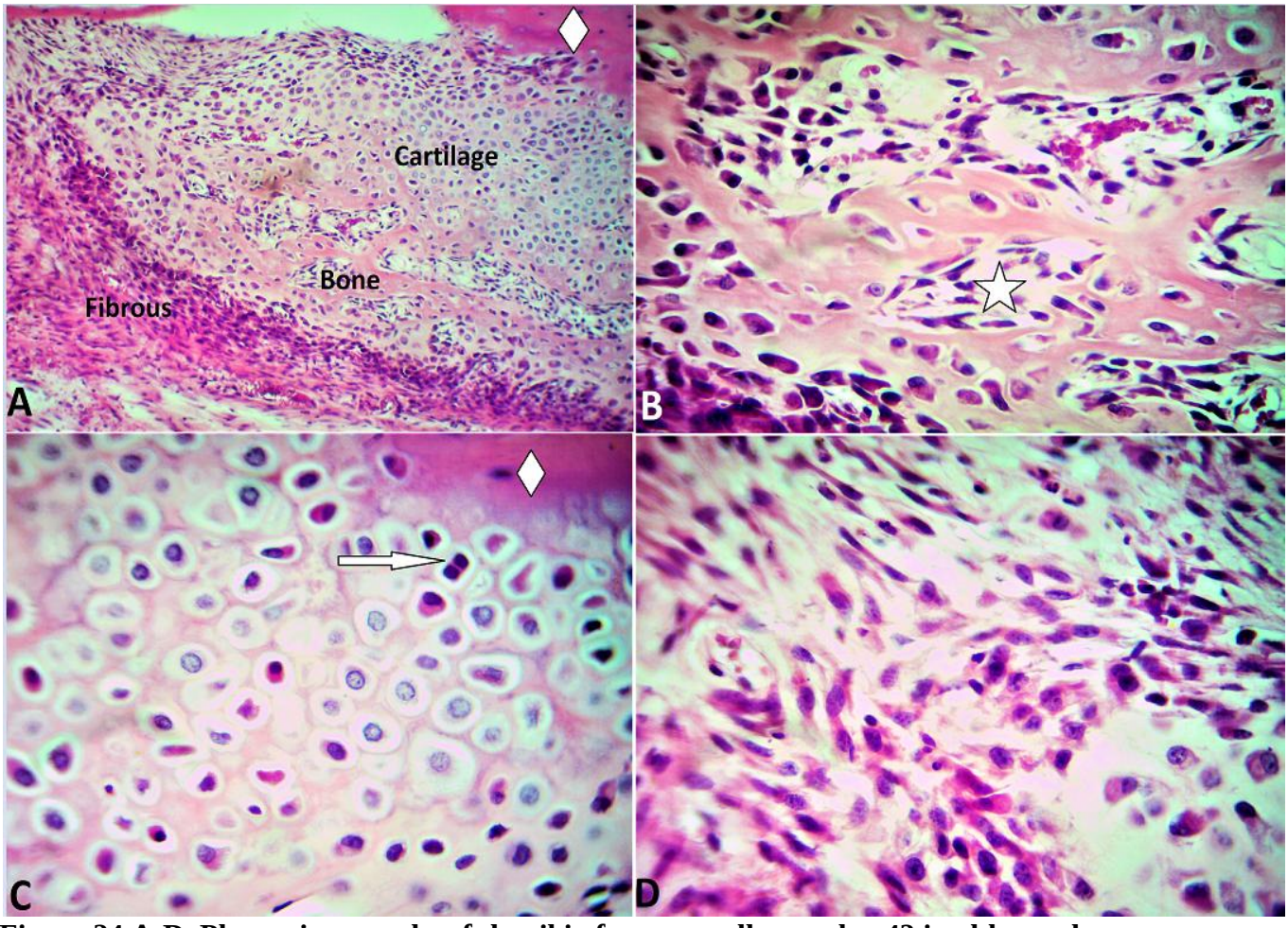


Figure 24 A-D. Photomicrographs of the tibia fracture callus on day 42 in old rats that received diclofenac [Haematoxylin and Eosin stain].

4.4.2 Histomorphometry of the Rat Tibia Fracture Callus

Comparing Different Age Groups

The older animals have a lower histological grade and proportion of bone on day 21. They also have a higher proportion of cartilage but these differences are not statistically significant. On day 42 the old animals have a lower histological grade and a higher proportion of cartilage. These differences are not statistically significant. The younger animals have a higher proportion of bone and this is statistically significant (Table 3).

Table 3. Histomorphometric parameters comparing old and young animals

	Histological Grade value(SD)		Proportion of Bone %(SD)		Proportion of Cartilage %(SD)	
	Young	Old	Young	Old	Young	Old
Day 21	5.45 (1.47)	4.70 (1.08)	34.48 (10.86)	32.41 (9.52)	23.43 (13.94)	29.12 (13.91)
Day 42	7.00 (1.34)	6.30 (1.08)	55.47 (9.82)	47.68 (9.98)*	16.62 (7.05)	17.40 (6.16)

*p=0.017 using the student T-test

4.4.3 Stereology of the Rat Tibia Fracture Callus Comparing Different Age Groups

The older animals have a higher osteoblast density on both days 21 and 42. These differences are however not statistically significant. On day 21 the younger animals have a higher chondrocyte density but by day 42 it is the older animals that have a higher chondrocyte density. This is however not statistically significant (Figure 25).

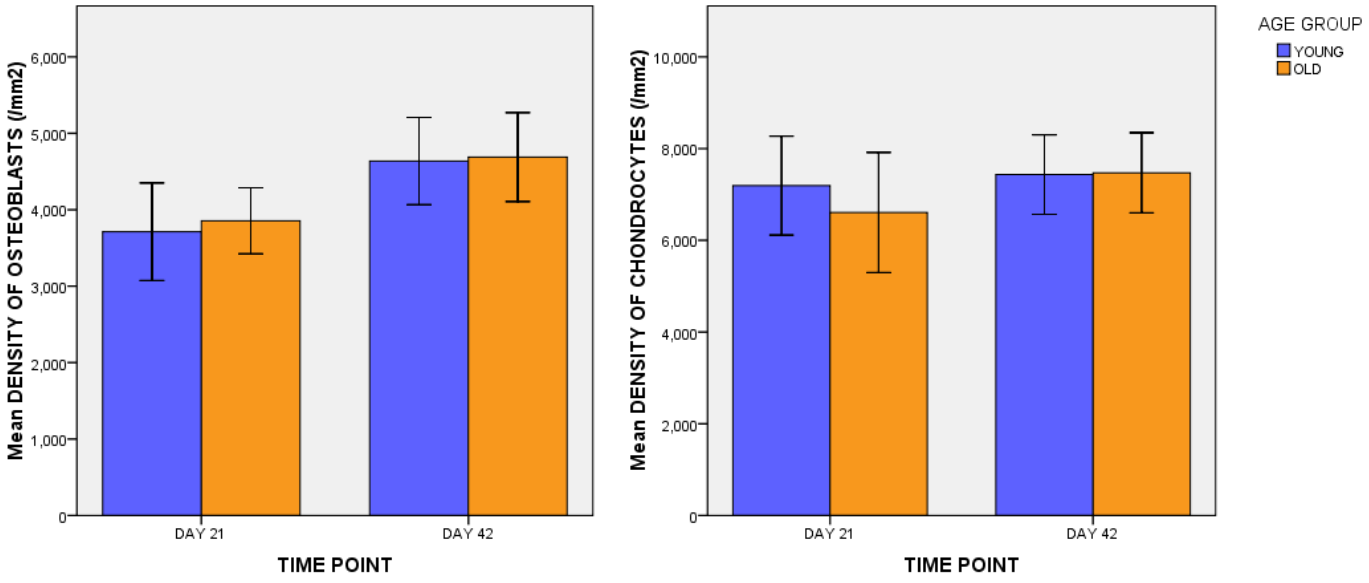


Figure 25. Graph of stereological parameters comparing old and young animals

4.5 DISTRIBUTION OF OSTEOCALCIN AND OSTEOPONTIN IN THE RAT TIBIA FRACTURE CALLUS

4.5.1 Distribution of Osteocalcin in the Rat Tibia Fracture Callus

Osteocalcin was expressed in the areas with immature bone (Figure 26A). At higher magnification, this was noted to be mainly localized to osteoblasts and the matrix surrounding these cells (Figure 26B). There was more robust staining for osteocalcin in the younger rats when compared to the older rats (Figure 27A). The staining in both cases was mainly confined to the areas with osteoblasts participating in bone formation (Figure 27B).

Figure 26 A-B. Photomicrograph of the rat tibia fracture callus showing immunostaining for osteocalcin at day 21 in young rats that received diclofenac

A: Fracture callus showing the fracture callus with the areas staining for osteocalcin (white arrows) [X100].

B: Higher magnification of the section in Figure 26A showing immature bone. Note the staining is localized in the cytoplasm of the osteoblasts and the surrounding matrix (white arrows) [X400].

C: Positive control from breast tissue [X100].

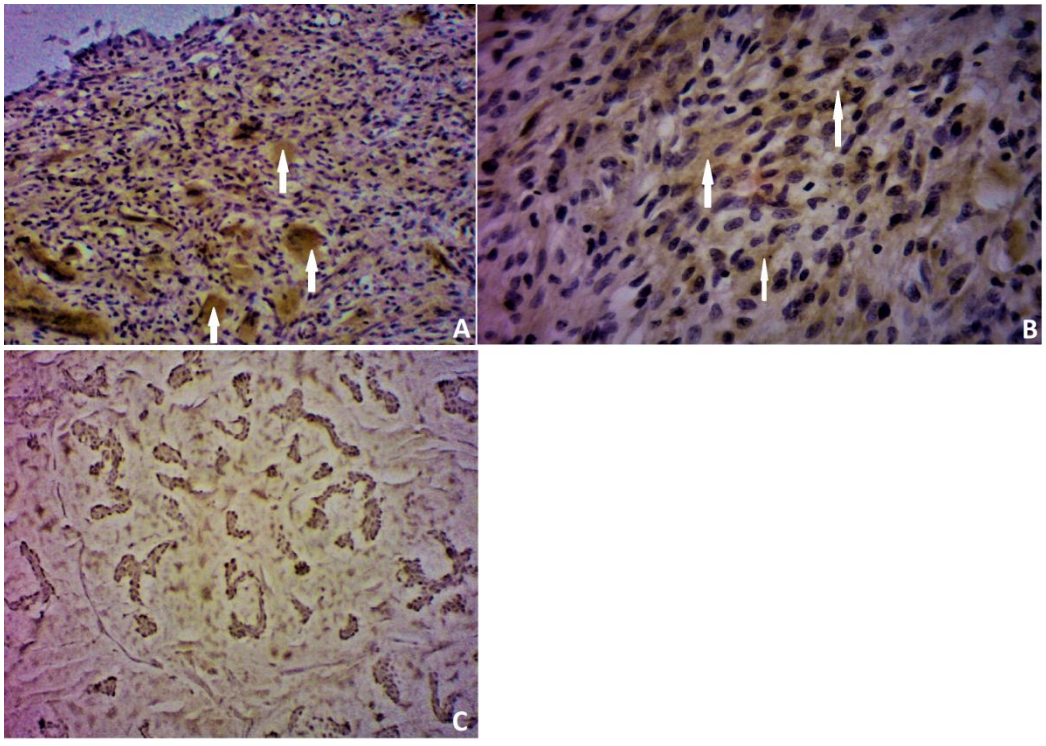


Figure 26 A-C. Photomicrograph of the rat tibia fracture callus showing immunostaining for osteocalcin at day 21 in young rats that received diclofenac

Figure 27 A-B. Photomicrograph of the rat tibia fracture callus showing immunostaining for osteocalcin in the young and old receiving diclofenac at day 21

A: Fracture callus showing the fracture callus of the young rat with the areas staining for osteocalcin (white arrows). Note the more intense staining in a larger proportion of callus [X100].

B: Fracture callus showing the fracture callus of the old rat with the areas staining for osteocalcin (white arrows). Note the relatively weaker staining in limited areas in the callus [X100].

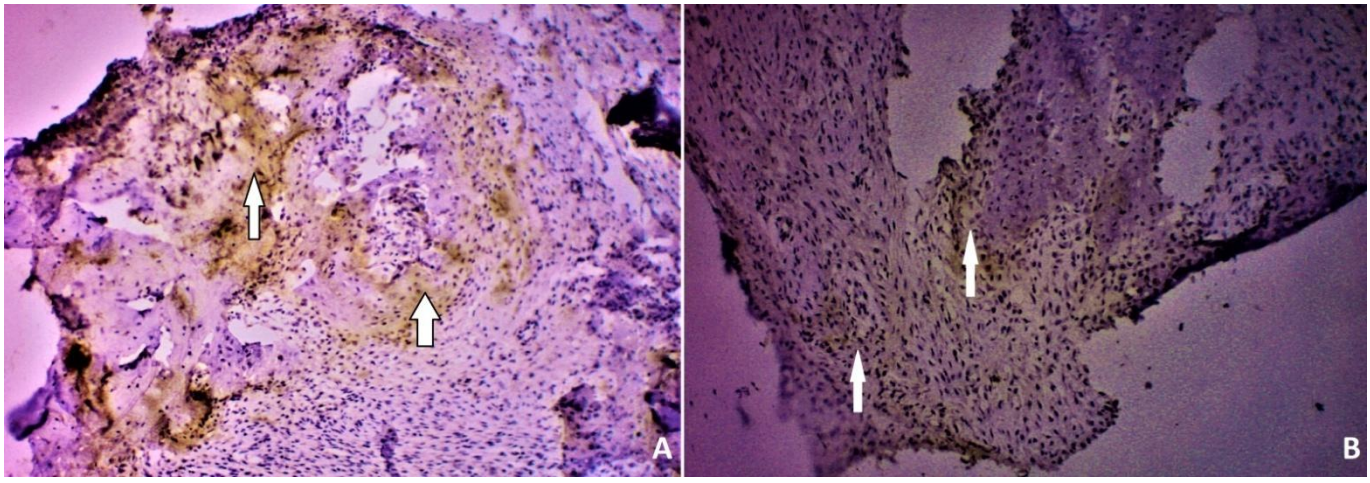


Figure 27 A-B. Photomicrograph of the rat tibia fracture callus showing immunostaining for osteocalcin in the young and old receiving diclofenac at day 21

4.5.2 Distribution of Osteopontin in the Rat Tibia Fracture Callus

Osteopontin was mainly distributed in the areas with bone and did not stain well in the areas with cartilage (Figure 28A). At higher magnification, this was noted to be mainly localized to osteoblasts and multinucleated osteoclasts. While the chondrocytes did not stain well, the matrix in the chondroid areas showed expression of the molecule (Figure 28B). There was more robust staining for osteopontin in the younger rats when compared to the older rats (Figure 29A). The staining in both cases was mainly confined to the areas with bone formation and remodeling (Figure 29B).

Figure 28 A-D. Photomicrograph of the rat tibia fracture callus showing immunostaining for osteopontin at day 21 in young rats receiving diclofenac

A: Fracture callus showing the fracture callus with the areas staining for osteopontin (white arrow) [X40].

B: Positive control from kidney tissue [X40].

C: Higher magnification of the section in Figure 28A showing cartilage areas. Notice the large cells in a lacuna with staining intracellularly (white arrow). Also, note the robust staining in the extra cell matrix (white star) [X400].

D: Higher magnification of the section in Figure 28A showing areas of immature bone. Note the large multinucleated osteoclasts with robust staining (white arrow). Also, note the osteoblasts which also show expression of osteopontin (white star) [X400].

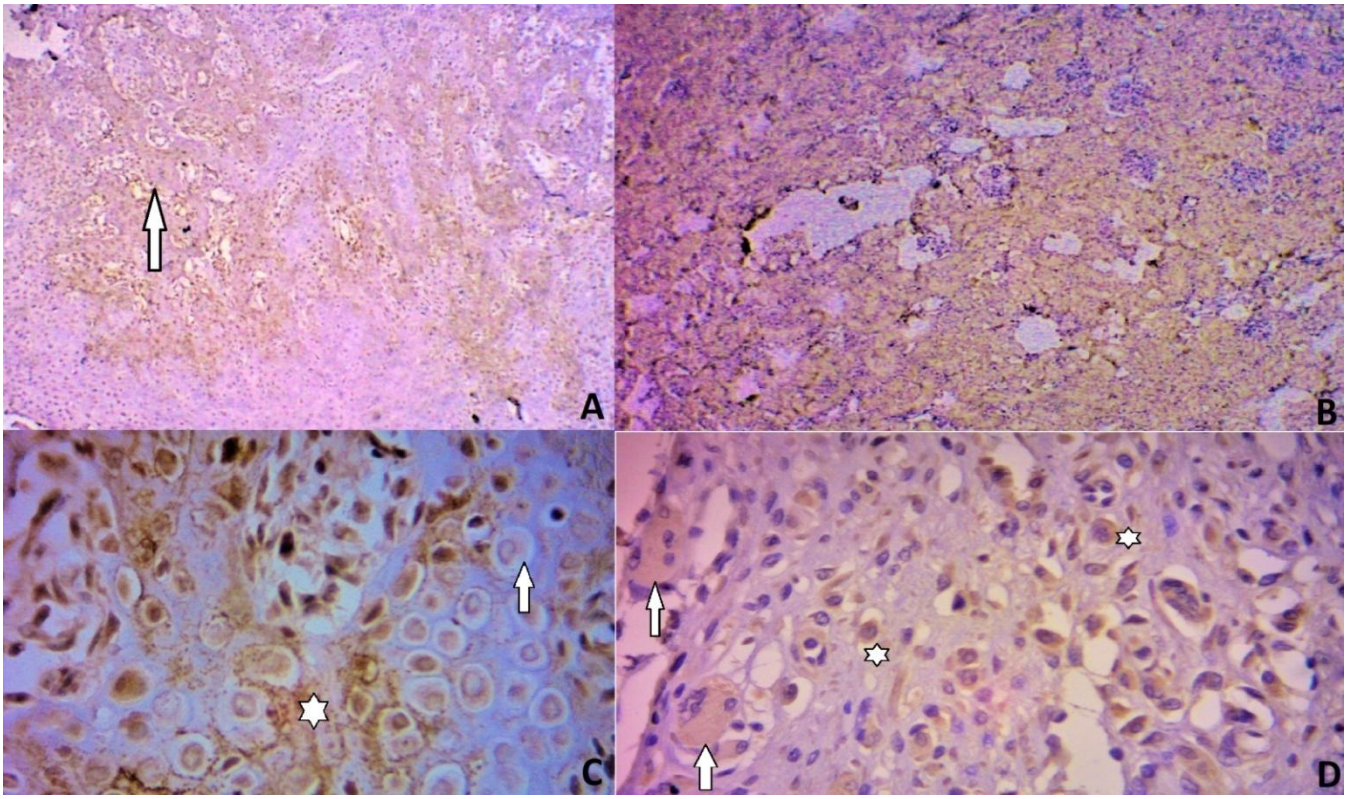


Figure 28 A-D. Photomicrograph of the rat tibia fracture callus showing immunostaining for osteopontin at day 21 in young rats receiving diclofenac

Figure 29A-B. Photomicrograph of the rat tibia fracture callus showing immunostaining for osteopontin at day 21 in the young and old receiving diclofenac

A: Fracture callus showing the fracture callus of the young rat with the areas staining for osteopontin (white arrow). Note the relatively larger volume of callus and no staining of the central cartilage areas [X100].

B: Fracture callus showing the fracture callus of the old rat with the areas staining for osteopontin (white arrow). Note the smaller volume of callus and the smaller area of staining [X100].

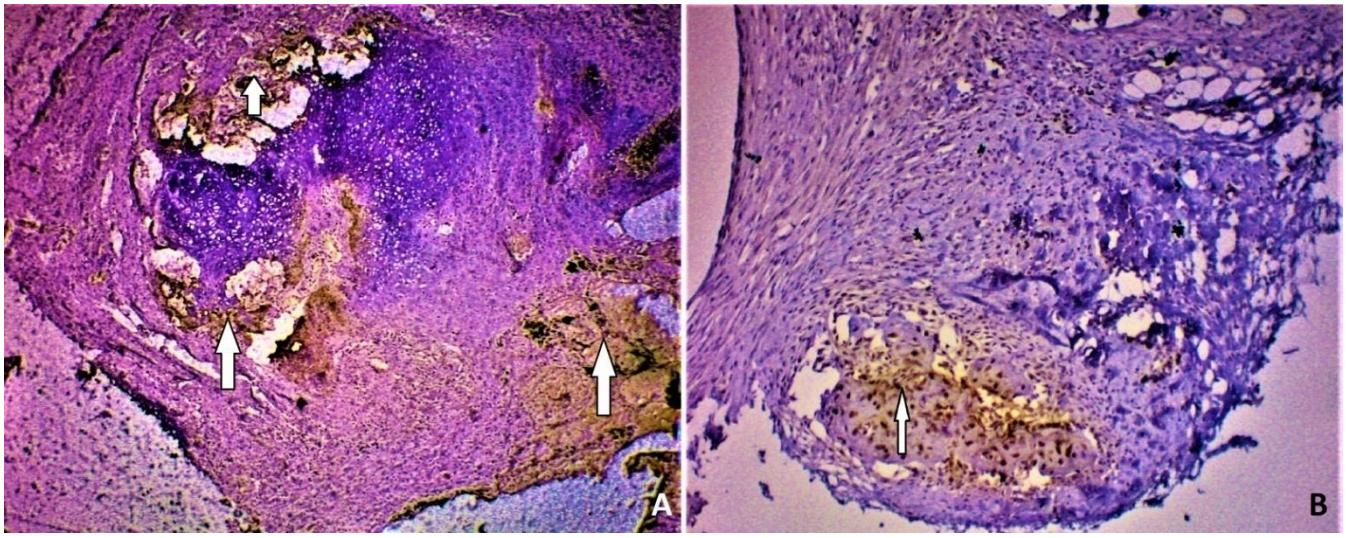


Figure 29 A-B. Photomicrograph of the rat tibia fracture callus showing immunostaining for osteopontin at day 21 in the young and old animals receiving diclofenac

4.6 RELATIONSHIP BETWEEN TYPE OF NSAID, TIMING OF DRUG USE, AGING, AND STRUCTURE OF THE RAT TIBIA FRACTURE CALLUS

4.6.1 Relationship Between Type of NSAID and Timing of Drug Use

Animals that received diclofenac after the stage of inflammation showed faster healing with better histological grades and higher proportions of bone, especially in the later stages of healing. This was more pronounced in the older animals compared to the younger ones. Animals receiving celecoxib early had higher cell densities compared to the other groups.

4.6.1.1 Histomorphometry of the Rat Tibia Fracture Callus Comparing Different NSAIDs and Timings

In the young animals, the diclofenac late group has the highest histological grade on day 21. The diclofenac early group shows the highest proportion of bone and the lowest proportion of cartilage. These differences are however not statistically significant. On day 42 the diclofenac late group has the highest histological grade while the diclofenac early group has the lowest proportion of cartilage. These differences are not statistically significant. The diclofenac late group has the highest proportion of bone and this is statistically significant (Table 4). Post hoc analysis reveals that the difference is between the diclofenac late and celecoxib early groups ($p=0.001$).

Table 4. Histomorphometric parameters comparing different NSAIDs administered at different timings in young rats

		Histological grade value(SD)		Proportion of Bone %(SD)		Proportion of Cartilage %(SD)	
		Early	Late	Early	Late	Early	Late
Day 21	Diclofenac	5.80 (1.79)	6.00 (1.58)	40.07 (8.41)	36.19 (10.54)	17.74 (9.79)	21.71 (7.49)
	Celecoxib	5.20 (1.09)	4.80 (1.48)	28.09 (5.13)	33.56 (16.04)	36.47 (18.63)	17.80 (11.31)
Day 42	Diclofenac	7.40 (0.55)	7.60 (0.55)	56.50 (6.90)	**64.56 (8.05)***	13.84 (3.70)	15.16 (5.87)
	Celecoxib	6.80 (0.84)	6.20 (2.39)	44.22 (4.47)	56.62 (7.85)	23.32 (8.34)	14.15 (6.49)

*p=0.003 using one-way ANOVA

In the older animals, the diclofenac late group has the highest histological grade, proportion of bone and proportion of cartilage on day 21. This is however not statistically significant. On day 42, the diclofenac late group has the highest proportion of cartilage but this is not statistically significant. The diclofenac late group also has the highest histological grade and proportion of bone and these differences are statistically significant (Table 5). Post hoc analysis reveals that the groups with differences are the diclofenac late and celecoxib early in the histological grade (p=0.029) and proportion of bone (p=0.045) respectively.

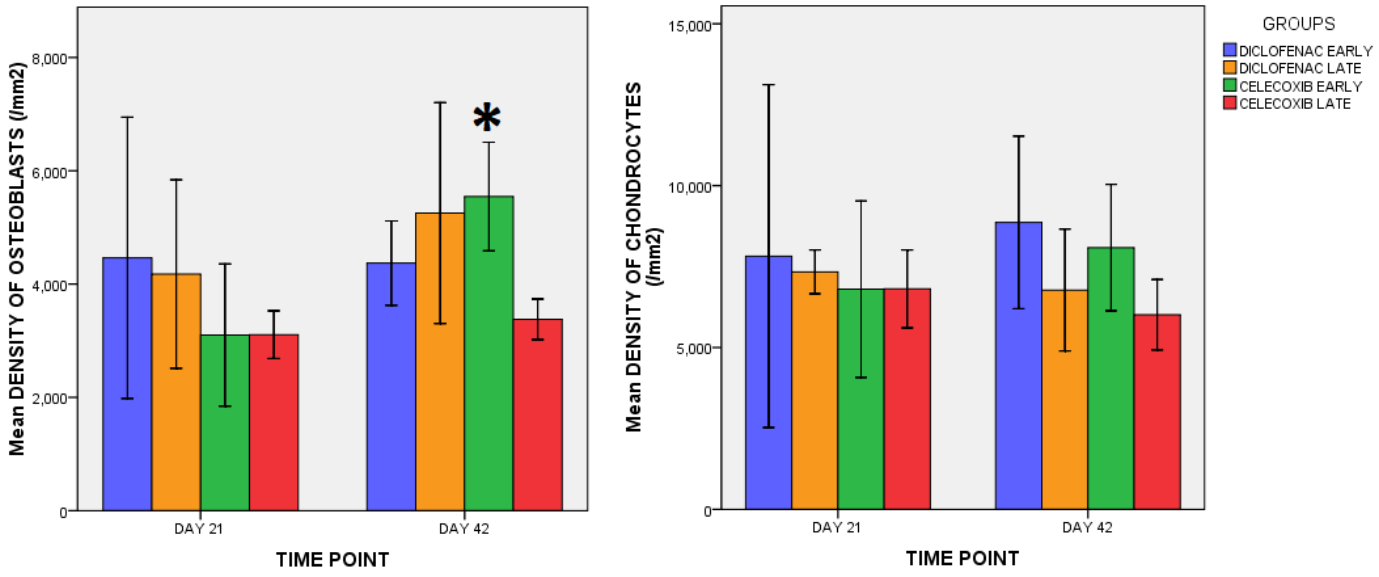
Table 5. Histomorphometric parameters comparing different NSAIDs administered at different timings in old rats

		Histological Grade value(SD)		Proportion of Bone %(SD)		Proportion of Cartilage %(SD)	
		Early	Late	Early	Late	Early	Late
Day 21	Diclofenac	4.80 (1.30)	5.00 (0.71)	35.42 (6.20)	36.65 (6.32)	20.90 (13.44)	38.33 (14.17)
	Celecoxib	4.20 (0.84)	4.80 (1.48)	23.85 (3.77)	33.71 (4.50)	28.29 (15.24)	28.97 (17.24)
Day 42	Diclofenac	6.60 (0.55)	**7.20 (0.84)***	51.53 (4.68)	**54.25 (2.92)****	17.41 (1.07)	20.99 (5.92)
	Celecoxib	5.40 (0.89)	6.00 (1.22)	38.49 (9.34)	46.46 (13.24)	15.80 (4.78)	15.41 (9.83)

*p=0.036 **p=0.049 using one-way ANOVA

4.6.1.2 Stereology of the Rat Tibia Fracture Callus Comparing Different NSAIDs and Timings

Among the young animals, the diclofenac early group has the highest osteoblast and chondrocyte density although these differences are not statistically significant on day 21. On day 42 the diclofenac early group has the highest chondrocyte density but the differences are not statistically significant. The celecoxib early group has the highest osteoblast density and this is statistically significant (Figure 30). Post hoc analysis reveals that the differences are between the celecoxib late group and the diclofenac late (p=0.027) and the celecoxib early (p=0.010) groups.

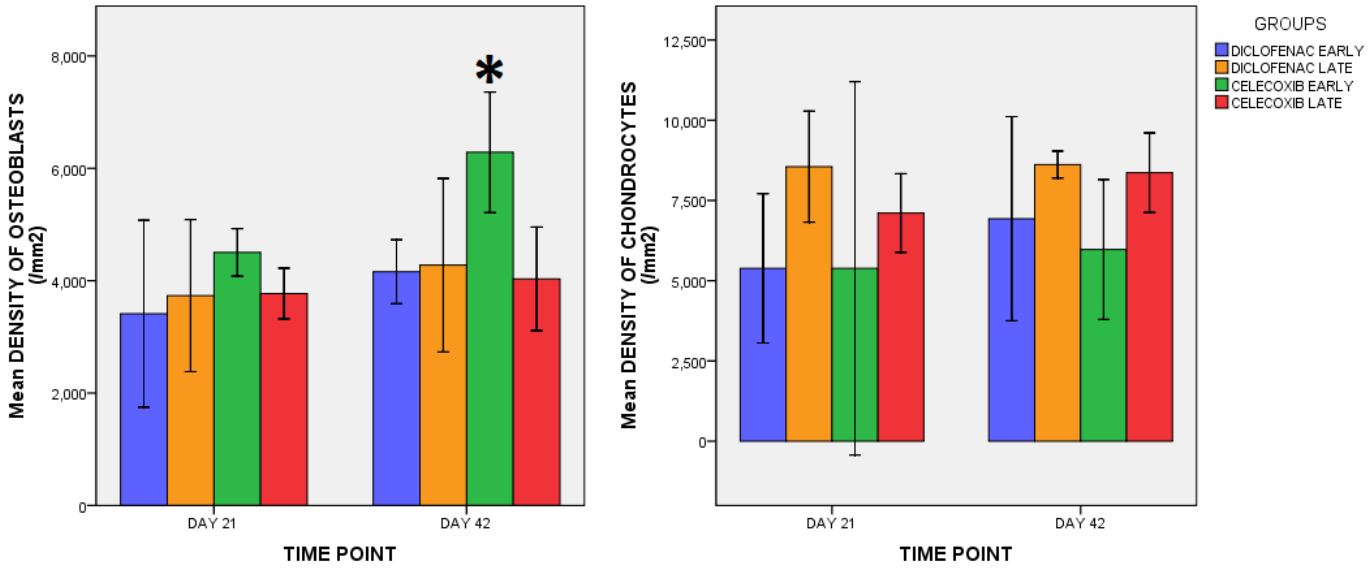


p<0.009 using one-way ANOVA

Figure 30. Graph of stereological parameters comparing different NSAIDs administered at different timings in young rats

Among the older animals, the celecoxib early group has the highest osteoblast density while the diclofenac late group has the highest chondrocyte density on day 21. These differences are not statistically significant. On day 42 the diclofenac late group has the highest

chondrocyte density although this is not statistically significant. The celecoxib early group has the highest osteoblast density and this is statistically significant (Figure 31). Post hoc analysis reveals that the differences are between the celecoxib early group and celecoxib late (p=0.004), diclofenac early (p=0.007), and diclofenac late (p=0.011) groups.



*p=0.002 using one-way ANOVA

Figure 31. Graph of stereological parameters comparing different NSAIDs administered at different timings in old rats

4.6.2 Relationship Between Type of NSAID and Aging

Analyzing the influence of both the type of NSAID and aging shows differences in the healing of the fracture callus with the old celecoxib group showing reduced healing. Young animals receiving diclofenac had higher histological grades and proportion of bone irrespective of the timing of drug administration. Older animals were also more likely to have higher cell densities compared to younger animals.

4.6.2.1 Histomorphometry of the Rat Tibia Fracture Callus Comparing Type of NSAID and Aging

In the animals receiving NSAIDs early, the young diclofenac group has the highest histological grade and the lowest proportion of cartilage on day 21. These differences are however not statistically significant. The old celecoxib group has the lowest proportion of bone and this is statistically significant. Post hoc analysis reveals differences between the old celecoxib and old diclofenac ($p=0.039$) and young diclofenac ($p=0.003$) groups. There are also differences between the young diclofenac and the young celecoxib group ($p=0.032$). On day 42 the young celecoxib group has the highest proportion of cartilage although this is not statistically significant. The young diclofenac group has the highest histological grade and proportion of bone with both differences being statistically significant (Table 6). The differences in the proportion of bone are revealed to be between the young diclofenac and young celecoxib ($p=0.045$) and old celecoxib ($p=0.003$) groups by post hoc analysis. The differences are also between the old diclofenac and old celecoxib groups ($p=0.031$). The differences in the histological grade are revealed to be between the young diclofenac and old celecoxib groups ($p=0.002$).

Table 6. Histomorphometric parameters comparing different NSAIDs administered early in various age groups

		Histological grade value(SD)		Proportion of Bone %(SD)		Proportion of Cartilage %(SD)	
		Young	Old	Young	Old	Young	Old
Day 21	Diclofenac	5.80 (1.79)	4.80 (1.30)	**40.07 (8.41)***	35.42 (6.20)	17.74 (9.79)	20.90 (13.44)
	Celecoxib	5.20 (1.09)	4.20 (0.84)	28.09 (5.13)	23.85 (3.77)	36.47 (18.63)	28.29 (15.24)
Day 42	Diclofenac	**7.40 (0.55)****	6.60 (0.54)	**56.50 (6.90)*****	51.53 (4.68)	13.84 (3.70)	17.41 (1.07)
	Celecoxib	6.80 (0.84)	5.40 (0.89)	44.22 (4.47)	38.49 (9.34)	23.32 (8.34)	15.80 (4.78)

*p=0.003 **p=0.004 ***p=0.003 using one-way ANOVA

In the animals receiving NSAIDs late, the old celecoxib group has the lowest histological grade while the young celecoxib has the lowest proportion of bone on day 21. This is not statistically significant. The young celecoxib group has the lowest proportion of cartilage and this is statistically significant. Post hoc analysis revealed differences between the old diclofenac and young celecoxib groups (p=0.046). On day 42 the young diclofenac group has the highest histological grade while the young celecoxib group has the lowest proportion of cartilage although this is not statistically significant. The old celecoxib group has the lowest proportion of bone and this is statistically significant (Table 7). Post hoc analysis revealed differences between the young diclofenac and old celecoxib groups (p=0.023).

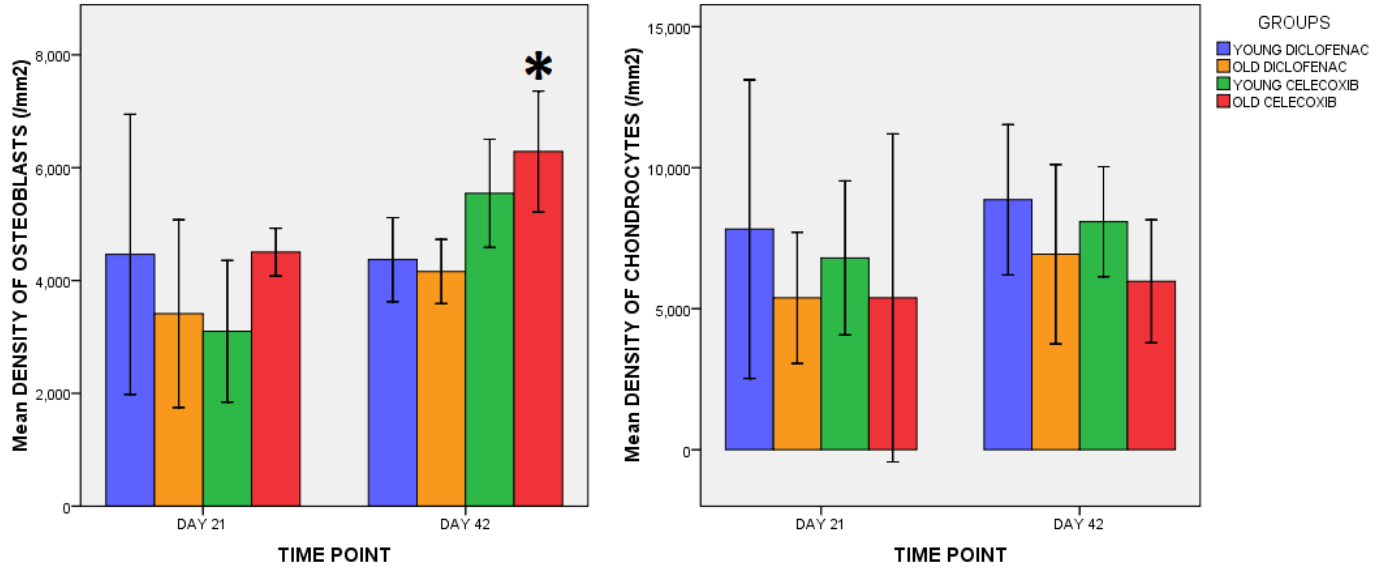
Table 7. Histomorphometric parameters comparing different NSAIDs administered late in various age groups

		Histological grade value(SD)		Proportion of Bone %(SD)		Proportion of Cartilage %(SD)	
		Young	Old	Young	Old	Young	Old
Day 21	Diclofenac	6.00 (1.58)	5.00 (0.71)	36.19 (10.54)	36.65 (6.32)	21.71 (7.50)	**38.34 (4.17)***
	Celecoxib	4.80 (1.48)	4.80 (1.48)	33.56 (16.04)	33.71 (14.50)	17.80 (11.31)	28.97 (17.24)
Day 42	Diclofenac	7.60 (0.55)	7.20 (0.84)	**64.56 (8.05)****	54.25 (2.92)	15.17 (5.87)	20.99 (5.92)
	Celecoxib	6.20 (2.39)	6.00 (1.22)	56.62 (7.85)	46.46 (13.24)	14.15 (6.49)	15.41 (9.84)

*p=0.049 p=0.037 using one-way ANOVA

4.6.2.2 Stereology of the Rat Tibia Fracture Callus Comparing Type of NSAID and Aging

Among the animals receiving NSAIDs early, the old celecoxib group has the highest osteoblast density while the young diclofenac group has the highest chondrocyte density on day 21. These differences are however not statistically significant. On day 42 the old diclofenac group has the highest chondrocyte density but this is not statistically significant. The old celecoxib group has the highest osteoblast density and this is statistically significant (Figure 32). Post hoc analysis reveals differences between the old celecoxib and young diclofenac ($p<0.001$), old diclofenac ($p<0.001$, and young celecoxib ($p=0.001$) groups. There are also differences between the young diclofenac and young celecoxib groups ($p=0.039$).

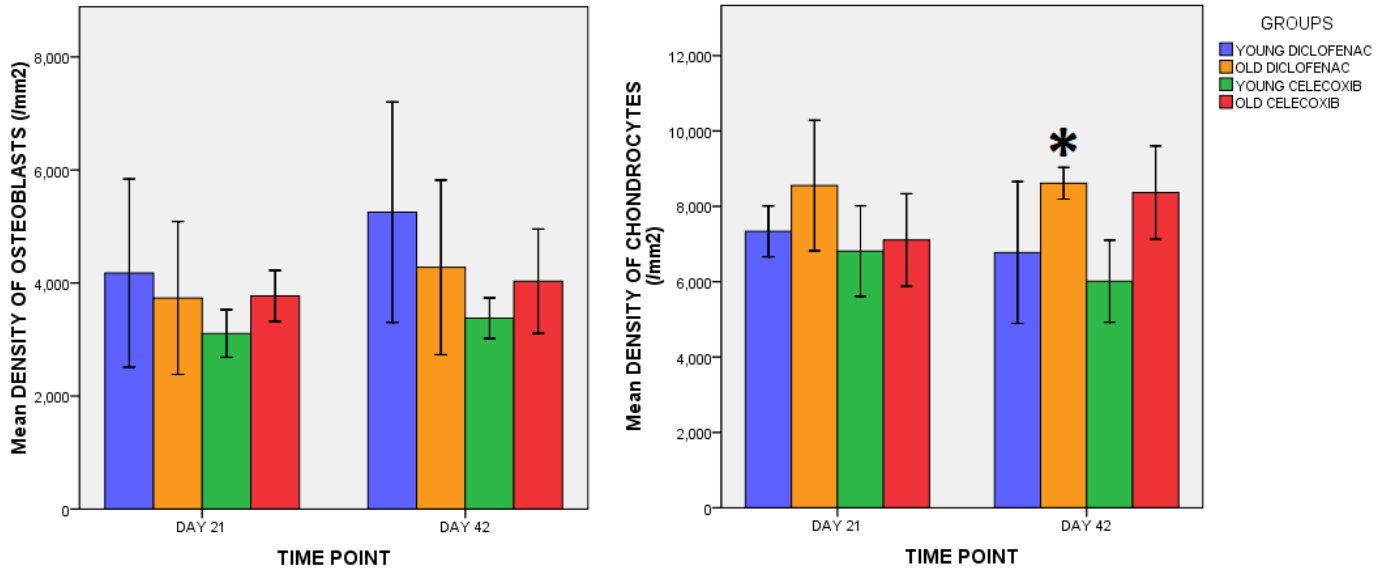


*p<0.001 using one-way ANOVA

Figure 32. Graph of stereological parameters comparing different NSAIDs administered early in various age groups

Among the animals receiving NSAIDs late, the young diclofenac group has the highest osteoblast density while the old diclofenac group has the highest chondrocyte density on day

21. This is not statistically significant. On day 42 the young diclofenac group has the highest osteoblast density but this is not statistically significant. The old diclofenac group has the highest chondrocyte density and this is statistically significant (Figure 33). Post hoc analysis reveals differences between the young celecoxib and old diclofenac groups (p=0.005).



p=0.002 using one-way ANOVA

Figure 33. Graph of stereological parameters comparing different NSAIDs administered late in various age groups

4.6.3 Relationship Between Aging and Timing of Drug Use

Evaluating the influence of both the aging and timing of drug use shows differences in the healing of the fracture callus with the young late group demonstrating better healing. Young animals that received the study medication late were more likely to have better histological grades and higher proportions of bone than older animals receiving medication early. The cell densities did not vary much across the groups studied.

4.6.3.1 Histomorphometry of the Rat Tibia Fracture Callus Comparing Aging and Timing of Drug Use

In the animals receiving diclofenac, the old early group had the lowest histological grade and proportion of bone on day 21, though this was not statistically significant. The young early group had the lowest proportion of cartilage and this was statistically significant (Table 8). Post hoc analysis revealed the differences between the old late group and the young early (p=0.015) and old early groups (p=0.042). On day 42, the young late group had the highest histological grade while the young early group had the lowest proportion of cartilage but these differences were not statistically significant. The young late group had the highest proportion of bone and this was statistically significant (Table 8). Post hoc analysis revealed the differences were between the young late group and the old early group (p=0.016).

Table 8. Histomorphometric parameters comparing different age groups and timings of NSAIDs in the diclofenac group

		Histological grade value(SD)		Proportion of Bone %(SD)		Proportion of Cartilage %(SD)	
		Young	Old	Young	Old	Young	Old
Day 21	Early	5.80 (1.79)	4.80 (1.30)	40.07 (8.41)	35.42 (6.20)	17.74 (9.80)	**20.90 (13.44)***
	Late	6.00 (1.58)	5.00 (0.71)	36.19 (10.54)	36.65 (6.32)	21.71 (7.49)	38.34 (4.17)

Day 42	Early	7.40 (0.55)	6.60 (0.55)	56.50 (6.90)	51.53 (4.68)	13.84 (3.71)	17.41 (1.07)
	Late	7.60 (0.55)	7.20 (0.84)	64.56 (8.05)	**54.25 (2.92)****	15.17 (5.87)	20.99 (5.92)

*p=0.013 **p=0.019 using one-way ANOVA

In the animals receiving celecoxib, none of the parameters were statistically different across the groups on day 21. On day 42 the young early group had the highest histological grade while the young late group had the lowest proportion of cartilage but these differences were not statistically significant. The young late group had the highest proportion of bone and this was statistically significant (Table 9). Post hoc analysis showed that the differences were between the young late group and the old early group (p=0.032).

Table 9. Histomorphometric parameters comparing different age groups and timings of NSAIDs in the celecoxib group

		Histological grade value(SD)		Proportion of Bone %(SD)		Proportion of Cartilage %(SD)	
		Young	Old	Young	Old	Young	Old
Day 21	Early	5.20 (1.10)	4.20 (0.84)	28.09 (5.14)	23.85 (3.78)	36.47 (18.63)	28.29 (15.24)
	Late	4.80 (1.48)	4.80 (1.48)	33.56 (16.04)	33.71 (14.50)	17.80 (11.31)	28.97 (17.24)
Day 42	Early	6.80 (0.84)	5.40 (0.89)	44.22 (4.47)	38.49 (9.34)	23.32 (8.34)	15.81 (4.78)
	Late	6.20 (2.39)	6.00 (1.22)	56.62 (7.85)	**46.46 (13.24)***	14.15 (6.49)	15.41 (9.84)

*p=0.046 using one-way ANOVA

4.6.3.2 Stereology of the Rat Tibia Fracture Callus Comparing Aging and Timing of Drug Use

In the animals receiving diclofenac, the young early and the young late groups had the highest osteoblast density on days 21 and 42 respectively, although this was not statistically significant. The old late and the young early groups had the highest chondrocyte densities on days 21 and 42 respectively, although this did not reach statistical significance (Figure 34).

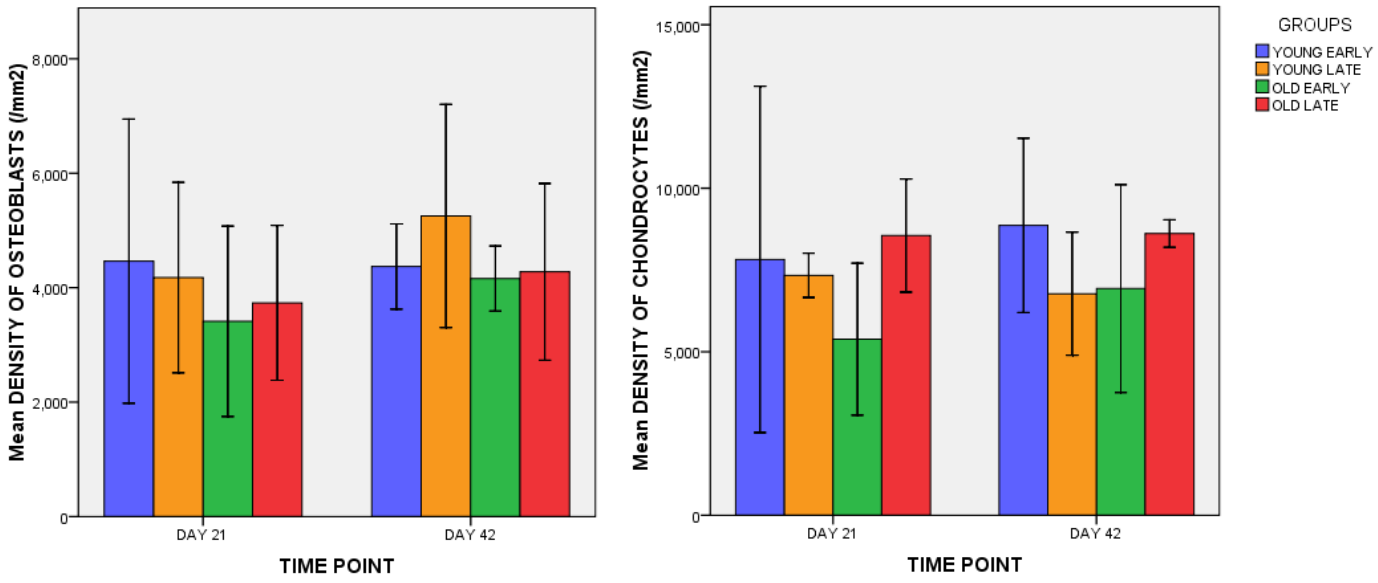
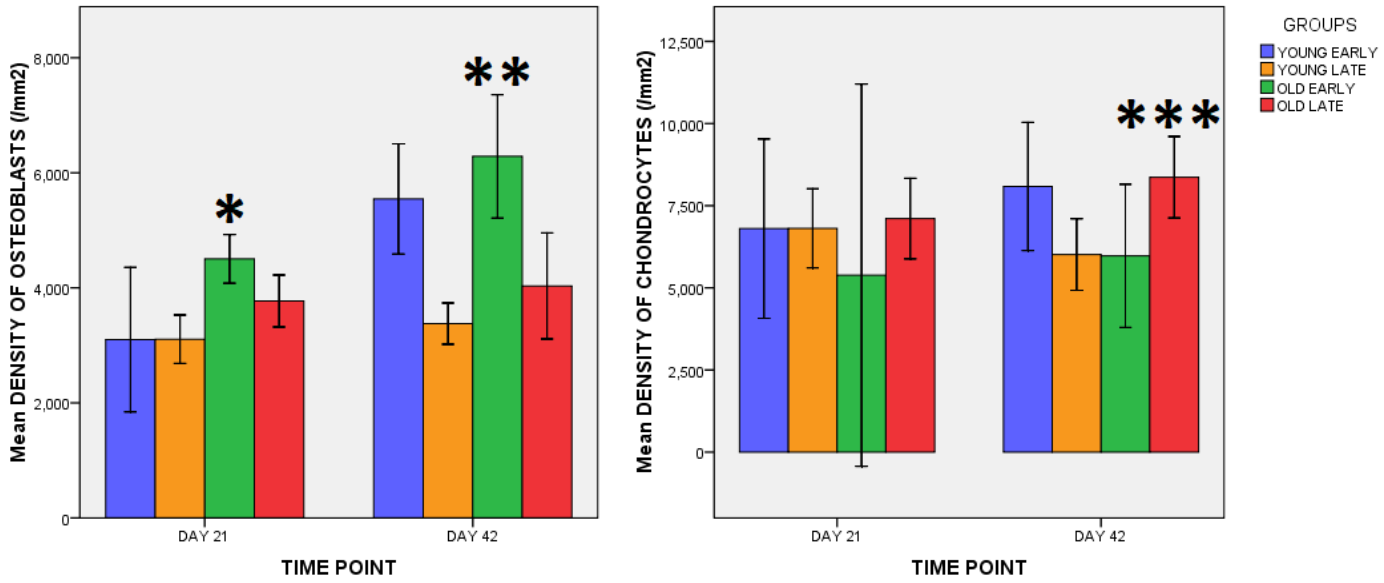


Figure 34. Stereological parameters comparing different age groups and timings of NSAIDs in the diclofenac group

In the animals receiving celecoxib, the old early group had the highest osteoblast density on both days 21 and 42 and this was statistically significant (Figure 35). Post hoc analysis revealed that the differences on day 21 were between the old early group and the young early ($p=0.008$) and old early groups ($p=0.008$). The differences on day 42 were between the old early group and the young late ($p<0.001$) and the old late ($p=0.001$) groups. There were also differences between the young early group and the young late ($p=0.001$) and the old late ($p=0.017$) groups. While the old late group had the highest chondrocyte density on both days, the differences on day 21 did not reach statistical significance like those on day 42 (Figure 35). Post hoc analysis did not reveal any group differences.

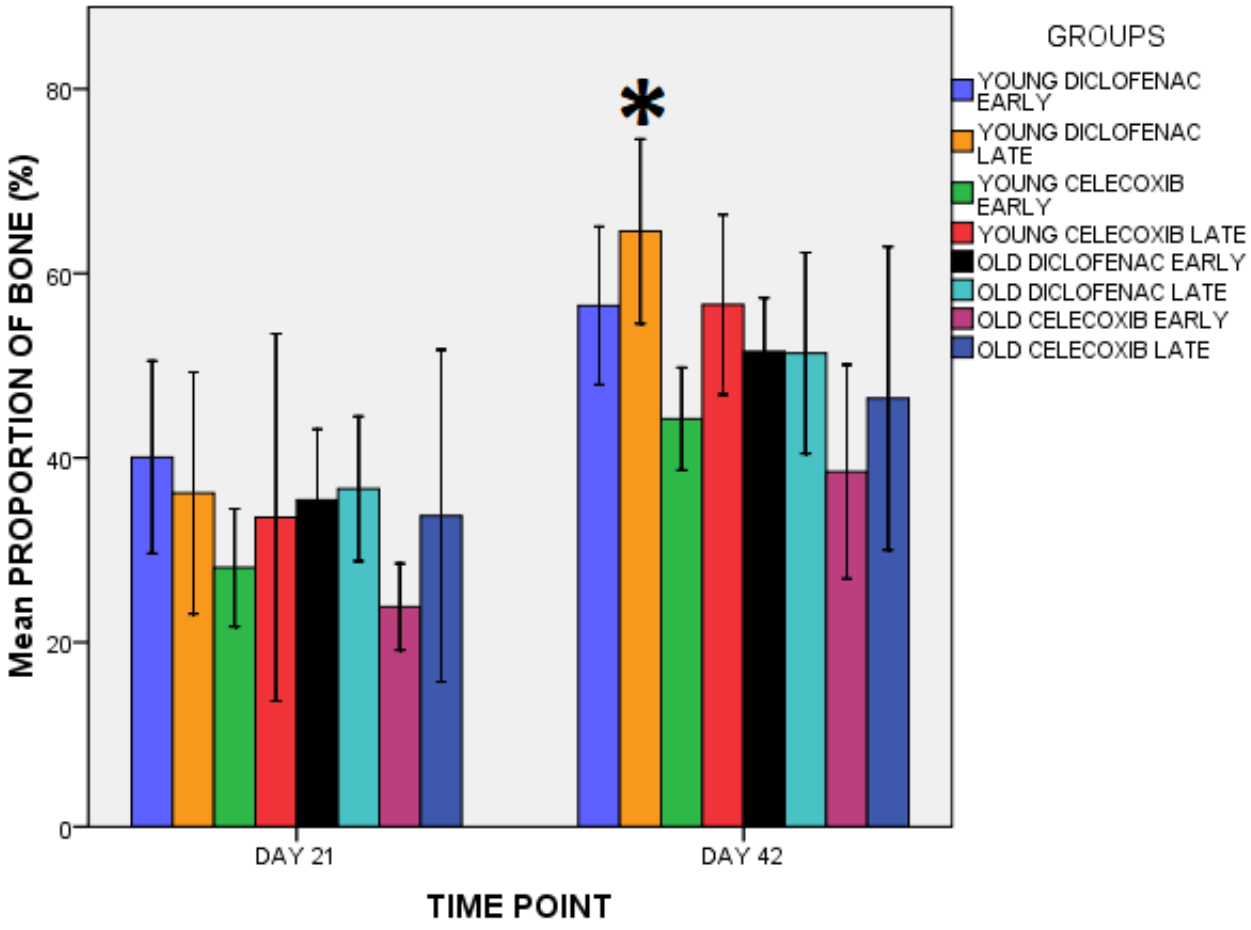


*p=0.005 **p<0.001 ***p=0.017 using one-way ANOVA

Figure 35. Stereological parameters comparing different age groups and timings of NSAIDs in the celecoxib group

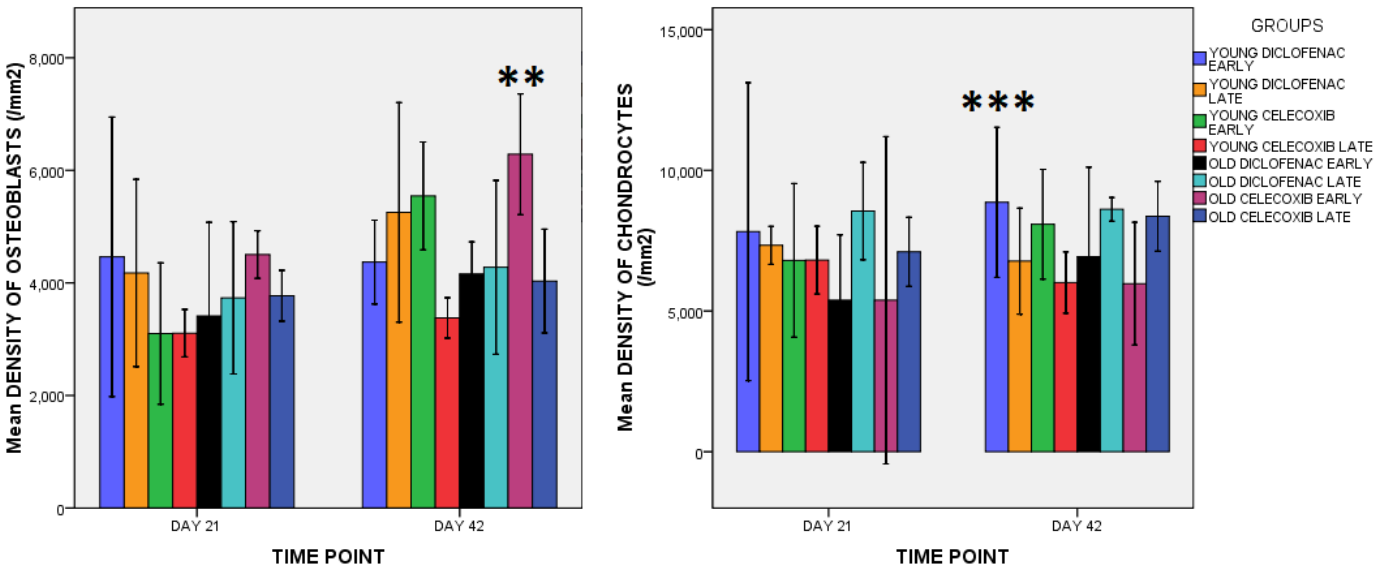
4.6.4 Combined Effect of NSAIDs, their Timing, and Aging

Comparing the influence of the type of NSAID, the timing of the drug administration, and aging on the fracture callus reveals notable differences across the groups. While the young and diclofenac groups had a higher histological grade and lower proportion of cartilage, the differences were not statistically significant. The young diclofenac late group had the highest proportion of bone on day 42 and this was statistically significant (Figure 36). Post hoc analysis found that the differences were between the young diclofenac late group and young celecoxib early ($p=0.005$), old celecoxib late ($p=0.018$), and the old celecoxib early ($p<0.001$) groups. There were also differences between the young diclofenac early group and the old celecoxib early group ($p=0.019$). The osteoblast and chondrocyte densities across the groups on day 21 did not show statistically significant differences. On day 42, the old celecoxib early group had the highest osteoblast density and this was statistically significant (Figure 37). Post hoc analysis reveals the differences between the old celecoxib early group and the young celecoxib late ($p<0.001$), old celecoxib late ($p=0.009$), the old diclofenac early ($p=0.016$), the old diclofenac late ($p=0.026$), and the young diclofenac early ($p=0.039$) groups. On day 42 the young diclofenac early group had the highest chondrocyte density and this was statistically significant (Figure 37). Post hoc analysis did not reveal any group differences.



*p<0.001 using one-way ANOVA

Figure 36. The proportion of bone comparing different NSAIDs, their timings, in different age groups



**p<0.001 ***p=0.029 using one-way ANOVA

Figure 37. Stereological parameters comparing different NSAIDs, their timings in different age groups

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 GENERAL FEATURES AND TEMPORAL CHANGES OF THE RAT TIBIA FRACTURE CALLUS WITH THE USE OF NSAIDS

5.1.1 General Features of the Rat Tibia

The rat tibia is subcutaneous and curved anteriorly. This is similar to the findings of other investigators and this makes it an ideal bone for designing a fracture model (Mustafy et al., 2018). The small size of the bone also makes it possible to have the entire fracture callus on one histological block making histological and stereological techniques easier.

5.1.2 Temporal Changes of the Rat Tibia Fracture Callus with the use of NSAIDs

From the time of induction of the fracture, the healing callus shows changes from a highly cellular fibrous material to an avascular cartilage stage that had an amorphous matrix. The cartilage was later replaced by immature highly vascular bone and later well-organized mature bone. The histological grade and the proportion of bone increased as the proportion of cartilage reduced.

5.1.2.1 Histomorphology of the Temporal Changes of the Rat Tibia Fracture Callus with the use of NSAIDs

In the initial fracture healing stages, the callus is highly cellular with equal proportions of mesenchymal fibrous tissue, cartilage, and bone. The callus changes to mainly bone in the

later fracture healing stages. The current study's findings are consistent with the literature and shows that the stages of fracture healing remain the same even in the setting of NSAID use (Janssen et al., 2017, Beil et al., 2011). Fracture callus is known to undergo various stages in the healing process from hematoma formation, inflammation, fibrovascular callus, bone formation, and remodeling (Bahney et al., 2019). Fracture healing also relies on various cells including the mesenchymal stem cells (MSCs) and immature myeloid cells (Levy et al., 2016). It is thus expected that the fracture callus morphology should undergo those changes. Bone in the healing callus is traditionally described to be formed from the apoptosis of the chondroid cells, vascular invasion, and replacement by the osteoblasts. Another mechanism of the formation of bone is the transdifferentiation of chondrocytes into osteoblasts (Zhou et al., 2014, Hu et al., 2017). Both of these mechanisms were likely at play in the healing of the rat tibia fracture callus. The reduction in cartilage and increased bone are necessary steps in the healing of fractures and higher proportions of bone are associated with better healing (Beil et al., 2011). These histological changes correlate with the increased mechanical strength and restoration of the form and function of the limb (Inglis et al., 2022).

5.1.2.2 Histomorphometry and Stereology of the Temporal Changes of the Rat Tibia Fracture Callus with the use of NSAIDs

The histological grade and proportion of bone increase as the proportion of cartilage reduces in the course of fracture healing. This is consistent with other studies in the literature (Zandi et al., 2017, Haffner-Luntzer et al., 2021) and indicates a gradual replacing of the fibrovascular and cartilaginous tissue with bone as is expected when the fracture heals. Tyler and colleagues were also able to document the gradual reduction of cartilage over time as the fracture heals (Tyler et al., 2022). While the osteoblast density increases over the period, the

chondrocyte densities are not different from baseline to 42. This is consistent with the findings of another study which found an increase in osteogenic cells (Dishowitz et al., 2013). This is, however, in contrast to a study on a mouse fracture model that demonstrated that the density of osteoblasts was similar over time (Brown et al., 2014). Another study also reported that the density of osteogenic cells remained stagnant over time as the fracture healed (Haffner-Luntzer et al., 2021). The animals in the current study were a mixture of those that received celecoxib and those that got diclofenac. While diclofenac may reduce osteoblast density, celecoxib has been shown to increase it (Chumpitaz-Cerrate et al., 2021, Costela-Ruiz et al., 2019). This may have resulted in the increasing osteoblast densities seen in the current study.

The new finding in the current study is that while the chondrocyte density increased over time, it did not reach statistical significance. This is in contrast to studies that found the number of chondrocytes reduced as the fracture healing progressed (Inada et al., 2022, Rundle et al., 2008). Chondrocyte proliferation has also been shown to reduce as the fracture heals (Oda et al., 2020). Half of the animals in the current study received celecoxib while the other half received diclofenac. Celecoxib has been found to reduce apoptosis in chondrocytes and this may stall the expected reduction in proliferation (Jeffrey and Aspden, 2007). It is hence likely that the reduced apoptosis leading to enhanced proliferation may have led to the chondrocyte numbers increasing, but not reaching statistical significance as seen in the current study. The mechanisms of this phenomenon deserve further research.

5.2 STRUCTURE OF THE RAT TIBIA FRACTURE CALLUS COMPARING DICLOFENAC AND CELECOXIB AND THEIR USE AT DIFFERENT TIMINGS

5.2.1 Histomorphology and Histomorphometry of the Rat Tibia Fracture Callus Comparing Diclofenac and Celecoxib used at Different Timings

The use of celecoxib is associated with lower histological scores, a lower proportion of bone, and higher proportions of cartilage. The use of NSAIDs early results in the persistence of mesenchymal fibrous tissue and lower proportions of bone especially in the later stages of fracture healing. The new overwhelming finding of the current study is the confirmation by multivariate analysis that the use of diclofenac after the first week of fracture healing resulted in a higher proportion of bone and histological grade than celecoxib administered during the first week when inflammation predominates.

The findings of the current study are consistent with findings in another study that has shown that celecoxib impairs the formation of bone in the fracture callus (Janssen et al., 2017). The study by Li and colleagues also showed less bone and increased fibrous tissue in animals that received celecoxib for four weeks (Li et al., 2013). The adverse effects of celecoxib are also likely to affect other musculoskeletal tissues other than bone. Celecoxib was associated with poor healing of rotator cuff injuries (Burns et al., 2020). Celecoxib has also been found to be

detrimental to the healing of bone-to-tendon interfaces (Constantinescu et al., 2019, Oh et al., 2018). The findings of better healing with the use of diclofenac in the current study are supported by the findings of other authors. A study on a rat model found that diclofenac did not affect fracture healing when compared to dexketoprofen, a non-selective COX inhibitor, and meloxicam, a selective COX-2 inhibitor (Inal et al., 2014). Locally administered diclofenac also did not result in impaired fracture healing in a rat model when compared to a placebo (Reich et al., 2020). Diclofenac also performed better than parecoxib a selective COX-2 inhibitor in a rabbit calvaria model (Cai et al., 2014).

The safety of diclofenac during fracture healing has, however, been called into question by some authors. A study of young rats found that diclofenac was associated with the persistence of cartilage and fibrous tissue in fracture callus (Bissinger et al., 2016). This study was however comparing diclofenac to prednisone and placebo. Diclofenac was also associated with poorer healing in a mouse model (Ramirez-Garcia-Luna et al., 2018). This study was however confounded by the use of carprofen, a COX-2 selective inhibitor, in the immediate post-operative period.

Clinical studies have also cemented the idea that non-selective COX inhibitors are relatively safe in fracture healing and this could arguably be the case with diclofenac. Ibuprofen did not disrupt fracture healing (Aliuskevicius et al., 2019), while ketorolac did not demonstrate any clinical or radiological fracture healing impairment (McDonald et al., 2018). The effect of NSAIDs on fracture healing is likely related to their inhibition of the COX-2 isoenzyme and the non-selective inhibitors cause less inhibition when compared to their COX-2 selective counterparts. The current study demonstrates that celecoxib may be worse than diclofenac and its effects may be more relevant in the clinical setting.

The impaired fracture healing with the use of celecoxib may not be extrapolated to all selective COX-2 inhibitors. Some investigators have not demonstrated any changes in fracture callus histomorphology with the use of parecoxib a selective COX-2 inhibitor (Hjorthaug et al., 2019). Although this study though limited by its use of medication for a short duration and by not determining the volume of the areas of cartilage, it demonstrates that impaired healing may not be attributable to all selective COX-2 inhibitors and each molecule deserves investigation. It also highlights that a drug likely needs to be administered for a longer period to have an effect.

NSAIDs especially celecoxib have been shown to have effects by action on the inflammatory system, the cell cycle, and the expression of certain key molecules related to fracture healing. Celecoxib is known to preferentially inhibit the COX-2 enzyme when compared to diclofenac (Lu et al., 2017). Fracture healing goes through several stages and the inflammatory stage relies on the COX enzyme to produce the inflammatory markers responsible for cell recruitment (Zheng et al., 2019, Bahney et al., 2019). Absence of this enzyme results in impaired fracture healing (Yukata et al., 2018). Inhibiting the enzyme, as with the use of NSAIDs would also impair fracture healing. The profound effects seen with celecoxib are likely to be seen with all selective COX-2 inhibitors and have been shown to result in delayed and non-union (George et al., 2020). Some of the adverse effects of selective COX-2 inhibitors on fracture healing may be independent of the inhibition of the enzyme. Parecoxib, a selective COX-2 inhibitor, has been shown to attenuate the inflammatory effects of leucocyte-rich platelet-rich plasma (LR-PRP) (Zhou et al., 2020). Celecoxib was also found to inhibit osteoblast differentiation independent of its COX inhibitory activity (Matsuyama et al., 2018).

NSAIDs have been shown to cause an arrest of the cell cycle of both animal and human MSCs, the first cells to be involved in fracture healing (Chang et al., 2007). This would lead to a lack of progression through the fracture healing stages and is likely to result in the persistence of mesenchymal fibrous tissue in the fracture callus as seen in the early healing period in the current study. Celecoxib may likely have a more profound effect on cell cycle inhibition than diclofenac. It has also been postulated that the effect of NSAIDs is to induce apoptosis of osteoblasts in the hypoxic setting seen after fractures (Liu et al., 2012). These effects are likely to reduce the number of cells available for the healing process thereby slowing it down.

The effects of celecoxib could also be due to its effect of reducing the expression of runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), and receptor activator of nuclear factor-kB ligand (RANKL) (Nagano et al., 2017, Ghalayani et al., 2014). This leads to a reduction in the osteoblast mediated mineralization and would result in a biomechanically weaker fracture union. This slowed healing and persistence of cartilage would also result in delayed union (Herbenick et al., 2008, Handoko et al., 2011). The effect of some NSAIDs on fracture healing has been postulated to be related to their effect on transforming growth factor beta 3 (TGF-β3), fibroblast growth factor 1 (FGF-1) expression, and the bone morphogenic protein (BMP) pathway (Spiro et al., 2010, Pountos et al., 2021).how does this lead to reduced bone? The effects of NSAIDs on fracture healing could also be related to the inhibition of chondrocyte hypertrophic differentiation and interaction with BMP-2 (Welting et al., 2011). Many NSAIDs including celecoxib have been shown to reduce the osteogenic differentiation of pre-osteoblasts and the activity of ALP (Hadjicharalambous et al., 2021). This enzyme is a key player in the complex process of osteoblast maturation and mineralization after a fracture (Golub and Boesze-Battaglia, 2007).

These changes result in slowing the progression of the stages of fracture healing, reduction in the proportion of bone, and increase in the proportion of cartilage resulting in delayed fracture union. The combined effect of all these mechanisms is delayed healing and it should caution clinicians who use celecoxib in the setting of musculoskeletal injury. It is also likely that other selective COX-2 inhibitors may have the same effect and they should also be avoided pending the results of future studies.

The current study's findings of poorer fracture healing with early administration of NSAIDs are similar to what has been reported by other investigators. Early administration of celecoxib has been implicated in poor fracture healing (Janssen et al., 2017, Li et al., 2013). Early administration of diclofenac was also associated with poorer fracture healing (Ramirez-Garcia-Luna et al., 2018). While this study was confounded by the co-administration of carprofen a selective COX-2 inhibitor, it demonstrates the possible adverse effects with either class of NSAID. Similar to the current study, early administration of ketorolac in a rabbit model showed impaired fracture healing (Ho et al., 1998). Although this study used a bone defect filled with demineralized bone matrix, ketorolac was associated with the persistence of calcified cartilage and delays in forming bone.

In contrast, however, early administration of rofecoxib a selective COX-2 inhibitor did not impair fracture healing in skeletally mature rabbits (Hak et al., 2011). While the difference with the current study could be attributed to different species used, it could also point to the varied effects of different molecules. The current results are also in contrast to a study on adult rats receiving parecoxib, a selective COX-2 inhibitor, that did not reveal any differences in fracture healing on day 28 with early drug administration (Hjorthaug et al., 2019). While this study differed from the current study in its short duration of drug use and including female rats, it highlights that the effects of NSAIDs may take some time to manifest and that

different molecules may have different effects. Most of the studies cited do not have a comparison group of animals receiving the medication late as is the case with the current study.

While there is a paucity of clinical studies investigating the timing of NSAID administration, some studies that have used the drugs from the early fracture period did not show impaired fracture healing. The current study's results are in variance with a clinical study that did not show any adverse effects with the early administration of ketorolac a non-selective COX inhibitor (Donohue et al., 2016). A clinical study that used ibuprofen failed to show any differences in fracture callus histomorphometry (Aliuskevicius et al., 2019). This study differed from the current study in that it used the drug for not more than one week, it co-administered paracetamol and tramadol and it was comparing the drug to a placebo. The study probably highlights that the effects of the drug would take time to effect and that different molecules would have varied effects emphasizing the need to investigate each. Early administration of ketorolac a non-selective COX-2 inhibitor did not result in any clinical or radiological fracture healing impairment (McDonald et al., 2018). While this study co-administered codeine and paracetamol and did not have a comparison group, the age of the participants was similar to the current study. There is a paucity of studies investigating the timing of drug administration with a comparison group of delayed NSAID administration and this could be the focus of future studies.

During the early healing period, the fracture callus goes through a stage of inflammation (Bahney et al., 2019). This inflammatory stage has been shown to last about a week and during this stage, the cell and humoral mechanisms necessary for fracture healing are set (Marsell and Einhorn, 2009). The cytokines released in this period play a role in the mobilization of mesenchymal stem cells (MSCs) which are later transformed into

chondrocytes in the fracture callus (Pajarinen et al., 2019). The cytokines have also been shown to aid the function of osteoblasts and osteoclasts in the fracture callus (Kurmis et al., 2012).

NSAIDs function by inhibiting the COX enzyme thereby reducing prostaglandin production and they can be classified according to their inhibition of the two isoenzymes of COX (Brune and Patrignani, 2015). The inducible COX-2 isoenzyme is the one responsible for the inflammatory pathway (Ozleyen et al., 2022), while COX-1 is found in almost all cells and regulates normal cell functions (Vane and Botting, 1996). Non-selective or traditional COX inhibitors like diclofenac have an almost equal effect on both isoenzymes while COX-2 selective inhibitors like celecoxib are more selective for the COX-2 isoenzyme (Kato et al., 2001).

When the histomorphologic and histomorphometric results are combined it is plausible that the use of celecoxib during the first week of fracture healing disrupts fracture healing when compared to diclofenac administered after the first week of inflammation. This disruption could then result in the persistence of cartilage in fracture callus, a lower proportion of bone, and poorer histological grades as seen in this current study. Selective COX-2 inhibitors and especially celecoxib should be avoided in the early fracture healing period and alternative analgesics are chosen. There is a paucity of studies investigating the timing of drug administration with a comparison group of delayed NSAID administration. This could be the focus of future studies.

5.2.2 Stereology of the Rat Tibia Fracture Callus Comparing Diclofenac and Celecoxib used at Different Timings

The osteoblast density did not vary with the drug used in the bivariate analysis. The new finding in the current study is the demonstration of higher osteoblast densities with early administration of NSAIDs and the confirmation on multivariate analysis that the use of celecoxib in the early fracture healing period results in higher osteoblast densities than with the use of diclofenac after the first week.

The findings of higher osteoblast densities with the use of celecoxib are similar to the findings of a recent rat model (Hajisadeghi et al., 2018). While this study was in a healing tooth socket model compared to the tibial fracture in the current study and used higher dosages of celecoxib, it is similar in using the same species and similar dosages of diclofenac. The findings are also similar to other studies that demonstrated a reduction in osteoblast density with the use of diclofenac (Krischak et al., 2007, Chumpitaz-Cerrate et al., 2021). Although the two studies are however constrained in that they compared diclofenac to placebo, they, however, demonstrate the effects of the drug on osteoblasts.

Diclofenac has been shown to exert its effect in several ways. It has been reported to affect the viability of pre-osteoblasts possibly resulting in the reduced osteoblast density seen in the current study (Hadjicharalambous et al., 2021). Diclofenac has also been shown to be cytotoxic to osteoblasts in a dose-dependent manner (Aguirre et al., 2021). Many NSAIDs and especially in increasing concentrations have been associated with arresting the cell cycle and increased apoptosis of osteoblasts which would result in reduced osteoblast densities (De Luna-Bertos et al., 2015). This justifies investigating each molecule to determine what effect it may have on these cells. Celecoxib on the other hand is protective of osteoblasts and this

could have resulted in the higher osteoblast densities seen in the current study (Costela-Ruiz et al., 2019). As the current study has also demonstrated that the use of celecoxib in the early fracture healing period was associated with poorer histomorphological and histomorphometric parameters, the increase in the cell density is unlikely to mitigate the effects of a lowered lower proportion of bone. Higher cell densities would also result in a reduced proportion of the matrix which bequeaths bone its structural properties resulting in biomechanically weak bone (Lin et al., 2020).

The chondrocyte densities did not vary with the drug used or with the timing of the drugs both on bivariate and multivariate analysis. This is a surprising finding as diclofenac may be cytotoxic to chondrocytes and may also inhibit chondrogenic differentiation of MSCs (Pountos et al., 2021, Nakamura et al., 2007). While the studies done on the effect of diclofenac on chondrocytes were conducted on cell cultures, one would expect a similar finding using histological techniques. The differences seen could be related to drug dosages. While oral diclofenac administration results in a larger volume of distribution and fluctuating drug levels with each dose, cell cultures tend to have a steady concentration on the cells (Yuan et al., 2017). Celecoxib on the other hand has been reported to have varying effects on chondrocytes. It has been associated with the reduction in the number of cells in the growth plate in a juvenile mouse model given the drug for 10 weeks (Caron et al., 2021). On the other hand, celecoxib has been shown not to affect chondrocyte numbers in a rabbit model (Welting et al., 2011) and reduce apoptosis of osteoarthritic chondrocytes (Cheleschi et al., 2021). While these conflicting findings in the literature could point to differences in methodology, animal species, and drug dosages, they also impress the need for future studies in this area to determine any differences conclusively. While there exist studies on the effects

of NSAIDs on chondrocytes in cell culture, there is a paucity of studies of the cells in fracture callus making it a rich ground for future studies.

5.3 STRUCTURE OF THE RAT TIBIA FRACTURE CALLUS COMPARING DIFFERENT AGE GROUPS

Younger animals show faster healing with rapid replacement of mesenchymal fibrous tissue and cartilage with bone. The histological grade increased at a faster pace in younger animals while the proportion of bone was higher when compared to the older animals. Older animals were more likely to have a cellular fracture callus than younger animals.

5.3.1 Histomorphology and Histomorphometry of the Rat Tibia Fracture Callus Comparing Different Age Groups

The younger rats show faster healing and the appearance of bone in the fracture callus much earlier when compared with older animals. The older animals have persistence of cartilage well into the later stages of fracture healing. In bivariate analysis, the younger animals have a higher proportion of bone and in the multivariate analysis, the young animals that received diclofenac have the highest histological grade and proportion of bone. After controlling for the drug used, younger animals receiving medication late have the highest proportions of bone. The new finding in this current study is the demonstration of a combined deleterious effect on fracture healing of the aging process, the use of celecoxib a selective COX-2 inhibitor, and its administration in the early fracture healing period.

The findings of less bone in older animals in the current study are in keeping with other investigators who have found less bone and more cartilage in the fracture callus of older animals (Clark et al., 2020, Burgers et al., 2013). The current results are also similar to another study that found the persistence of cartilage and the later appearance of bone in older

animals (Lu et al., 2005). In another study, 52-week-old female rats were found to have more cartilage and collagen fibers compared to their 12-week-old counterparts that had more bone on histology (Mehta et al., 2010) while another study found poorer healing and delayed callus formation in sixty-week-old mice when compared to five-week-old ones (Matsumoto et al., 2016). The persistence of cartilage and reduction of bone would result in biomechanically weaker bone. A study comparing young and old female rats showed a reduction in the mechanical properties of fracture callus in the older group (Strube et al., 2008). Yukata and colleagues also note a difference in the proportion of bone between young and aged mice although this did not reach statistical significance (Yukata et al., 2014). While they used a different species and their animals were only one year old, they were able to demonstrate that aging slows down fracture healing.

The current study's findings of no difference in the proportion of cartilage are similar to the findings of other investigators (Lopas et al., 2014). While the authors used 25-month-old mice as compared to the current study which used 15-month-old rats, they were able to demonstrate similar proportions of cartilage. A recent study comparing three-month and eighteen-month-old mice without the administration of NSAIDs found that the proportion of cartilage was not different between the groups but the proportion of bone was lower in the older age group (Clement et al., 2022).

These animal studies are in keeping with clinical studies that have shown that while older patients have impaired fracture healing, children rarely have fracture healing impairments. Children were shown not to suffer healing retardation with the use of NSAIDs (Nuelle et al., 2020). Another clinical study found that ibuprofen was not associated with impaired fracture healing in children (DePeter et al., 2016). Ketorolac was also not associated with increases in the rates of non-union among children undergoing operative fracture fixation (Kay et al.,

2010). The finding that NSAIDs are safe in children is cemented by a recent systematic review (Stroud et al., 2022). Administration of ketorolac a non-selective COX-2 inhibitor also resulted in clinically evident fracture healing impairment in an elderly population (McDonald et al., 2018). While this study did not have a comparison group with a different NSAID, the age of the participants was similar to the current study.

The current study's findings are, however, in contrast to a study that found no difference in the proportion of bone in a mouse model (Lopas et al., 2014). The difference with this study could be due to the difference in species and that they used much older animals with an equivalent human age of 70-85 years compared to 50 years in the current study.

In contrast, other clinical studies failed to find a relationship between aging and non-union (Zura et al., 2017, Tanner et al., 2019). A study of elderly individuals did not show any differences in fracture callus histomorphometry with the use of ibuprofen a non-selective NSAID (Aliuskevicius et al., 2019). While this clinical study compared the drug to a placebo, it differs from the current study in its lack of a control group of young patients, the short duration of drug use, and co-administration with paracetamol and tramadol.

Although young animals fare better than older ones, caution is still needed when using NSAIDs in the setting of a fracture. Several animal studies have noted impaired healing when celecoxib was used in young animals (Janssen et al., 2017, Li et al., 2013). While the difference in the rabbit study by Jansen and colleagues could be attributed to the use of a different species, it could also be because the authors used a drilled cortical defect as opposed to a fracture. This may have resulted in a smaller volume of the fracture hematoma and possibly a poorer mobilization of the MSCs required for fracture repair. The study by Li and colleagues also differed from the current study because they compared the celecoxib group to a placebo group and did not measure the areas in the fracture callus occupied by cartilage.

Despite these shortcomings, the studies highlight the need for caution when using selective COX-2 inhibitors like celecoxib, even in the young. Clinical studies also reinforce caution as non-union was noted among children undergoing operative fracture fixation with the use of NSAIDs (Kay et al., 2010). The use of celecoxib in older individuals will lead to impaired healing, especially when administered in the first week after a fracture, and should therefore be avoided.

Several mechanisms have been identified that may explain this reduced fracture healing potential including senescing cells, altered molecular mechanisms, disordered inflammation, and reduced responsiveness to mechanical stimuli. The reduced healing potential in older animals may be related to the senescence of the osteoprogenitor cells (Lopas et al., 2014, Yukata et al., 2014). Older rats have been found to have a lower healing potential and this has been reversed by implantation of bone marrow from younger rats (Baht et al., 2015). This suggests that the culprit is the senescence of the osteoprogenitor cells. There is also evidence that older rats have increased fatty degeneration of the bone marrow and reduced osteogenic potential (Leucht et al., 2013).

The delayed healing in older animals has also been linked to reduced expression and activity of the sonic hedgehog (SHH) protein (Matsumoto et al., 2016). Other investigators have related the reduced osteogenic potential of elderly rats to is related to the Wingless/Integrated (WNT) signaling (Leucht et al., 2013). Aging has been associated with changes in over 120 genes and a reduction in the ability of MSCs to respond to mechanical stimuli (Ode et al., 2014).

Another theory for delayed healing in aging animals is the senescence of the inflammatory cells. Aging rats showed better fracture healing when they were transplanted with the inflammatory cells from young mice (Xing et al., 2010). The slower healing of fractures with

aging could be due to an imbalance between the osteogenic and osteoclastic cells. Mehta and colleagues related the reduced healing potential in the elderly to increasing numbers of osteoclasts (Mehta et al., 2010).

The healing of fractures is greatly dependent on the mechanical stimuli received during the healing period and these forces are transmitted to the cells in the fracture callus (Augat et al., 2020). Advancing age is, however, associated with an altered cellular mechanoresponse resulting in the cells in the fracture callus responding less to the mechanical stimuli in the fracture leading to altered cell differentiation (Borgiani et al., 2019). The result of this is a slower progression of the cells from MSCs to the bone-laying osteoblasts and a subsequent slowing of the fracture healing processes. Aging reduces the healing potential through several mechanisms and this is worsened by the use of NSAIDs especially the selective COX inhibitors like celecoxib. This has to raise the threshold for the use of celecoxib in this age group and different analgesics should be considered.

The finding of high levels of cartilage in young animals receiving NSAIDs early should also be noted. While it illustrates that early administration of NSAIDs is detrimental even in the young, it seems the effects do not last into the later stages of fracture healing. This is likely the result of mitigation by the robust healing mechanisms of young individuals. It should, however, raise the threshold for early administration of NSAIDs even in the young. There is a paucity of studies investigating the timing of drug administration with a comparison group of delayed NSAID administration. This could be the focus of future studies.

5.3.2 Stereology of the Rat Tibia Fracture Callus Comparing Different Age Groups

There was no difference in the osteoblast densities between the younger and older rats on bivariate analysis. Multivariate analysis showed high osteoblast densities in older animals that received celecoxib but only in the animals that received the drug in the early fracture healing period. This was confirmed even after controlling for the drug used. The findings on osteoblast densities are in variance with a study that showed lower densities in twenty-four-month-old mice when compared to their six-month-old counterparts (Kim et al., 2017). Another study on fractured ribs in a mouse model showed higher osteoblast densities in the younger age group (Matsumoto et al., 2016).

The difference between the current and the cited studies could be due to the effect of early drug administration and the effects of NSAIDs on the chronic inflammation seen in aging. The current study had previously demonstrated that early administration of celecoxib resulted in increased osteoblast densities, a finding that was confirmed by another study in the literature (Hajisadeghi et al., 2018). This phenomenon is mainly attributed to the protective properties of celecoxib on osteoblasts (Costela-Ruiz et al., 2019). As the higher osteoblast densities were only seen in the older animals that received celecoxib in the early fracture healing period and not those that received the drug after the first week of fracture healing, it is plausible that the timing of the drug was a factor influencing the findings.

Aging has been shown to lead to a reduction in the capacity to respond to stressors and an increase in the pro-inflammatory status, a phenomenon that has been named inflammaging (Franceschi et al., 2000). This inflammaging has been shown to reduce the number of osteoprogenitor cells that are necessary for fracture healing (Josephson et al., 2019). The use

of NSAIDs has been associated with the reversal of some of the effects of inflammaging and could lead to an increase in osteoblast density (Fielder et al., 2020). Although both diclofenac and celecoxib have been shown to reduce inflammaging in multiple tissues (Rivers-Auty et al., 2020), celecoxib has been shown to have effects on the musculoskeletal system (Cai et al., 2022). This may result in the animals that received celecoxib having a more profound reduction in inflammaging, leading to a higher osteoblast density as seen in the current study. The higher osteoblast density is unlikely to result in improved healing in older animals. The current study has previously demonstrated that older animals especially those that received celecoxib had lower proportions of bone in the fracture callus signaling slower healing. The aging process is also associated with a decline in the function of the reparative cells, and the increase in cell density does not result in faster healing of the fracture (Kassem and Marie, 2011). The reduction in the proportion of bone and the function of cells would still result in slower healing in older animals. There is a paucity of studies on the cell densities in the fracture callus of the aging rat and this could be the focus of future studies.

While there was no difference in the chondrocyte density between the younger and older animals on bivariate analysis, multivariate analysis showed higher chondrocyte densities in the older animals that received diclofenac but only in the animals that received the medication after the early fracture healing period. After controlling for the type of drug used, older animals receiving medication after the early fracture healing period had a higher chondrocyte density, especially with the use of celecoxib. The overarching theme in these results is that older animals were more likely to have higher chondrocyte densities irrespective of drug and timing.

Aging has been associated with delays in chondrocyte differentiation, maturation, and vascular invasion of cartilage, thereby prolonging the lifespan of the chondrocytes and

increasing their density (Lu et al., 2005). This could explain the higher chondrocyte density seen in the older animals in the current study. Aging has also been reported to cause a slowing of the trans-differentiation of chondrocytes to osteoblasts (Huang et al., 2020). This would result in disproportionately larger numbers of chondrocytes in the older animals as was demonstrated in the current study.

5.4 COMBINED EFFECT OF NSAIDS, THEIR TIMING, AND AGING ON THE HEALING RAT TIBIA FRACTURE CALLUS

When all the parameters studied are considered, older animals receiving celecoxib in the first week after a fracture had the lowest proportion of bone while younger animals receiving diclofenac had the highest. This was especially prominent in the later stages of fracture healing. The use of celecoxib in the early fracture healing period in older animals was associated with the highest osteocyte density. The use of celecoxib has been demonstrated to be detrimental to fracture healing (Janssen et al., 2017) and aging also results in slower healing (Clark et al., 2020). The early administration of NSAIDs has also been reported to result in poorer healing (Ramirez-Garcia-Luna et al., 2018). The current study's findings while new are thus not surprising. The current study has demonstrated the possible adverse effects of the use of celecoxib in older people with fractures and that these effects are worse if the medication is given in the first week after a fracture. This should prompt the use of different medications to manage pain in these individuals.

Older animals have been shown to have higher osteoblast counts, especially with the use of celecoxib. The process of aging increases chronic inflammation which reduces the number of osteoprogenitor cells necessary for fracture healing (Josephson et al., 2019). However, celecoxib is effective in mitigating the musculoskeletal effects of inflammaging (Cai et al., 2022). The combined effect of this is the increased osteoblast counts seen in the current study. While the cell number is increased, aging results in a decline in the function of the cells, and hence the fractures do not heal faster (Kassem and Marie, 2011). As the mechanical properties of bone are from its matrix, the highly cellular fracture callus with lower proportions of bone would be biomechanically weak (Lin et al., 2020).

5.5 DISTRIBUTION OF OSTEOCALCIN AND OSTEOPONTIN IN THE RAT TIBIA FRACTURE CALLUS

Osteocalcin and osteopontin were mainly distributed in the areas with actively forming bone and younger animals had more robust staining than the older ones. While chondrocytes did not stain well, osteoblasts and osteoclasts had robust staining with osteopontin.

5.5.1 Distribution of Osteocalcin in the Rat Tibia Fracture Callus

Osteocalcin was found mainly in the areas with osteoblasts laying down bone. Younger rats had more intense staining in a larger area when compared to older rats. The current study's findings of staining in the areas occupied by bone are similar to other findings in the literature (Luvizuto et al., 2010). Osteocalcin is reportedly expressed in chondrocytes intracellularly and this could represent trans-differentiated chondrocytes functioning as osteoblasts (Hu et al., 2017). Osteocalcin is necessary for the proper mineralization of bone after the osteoblasts have laid down the matrix (Moriishi et al., 2020). The more intense staining in the younger rats suggests that we should expect better mineralization and hence better healing in the young as was demonstrated in the current study's histomorphologic and histomprphometric results.

5.5.2 Distribution of Osteopontin in the Rat Tibia Fracture Callus

Osteopontin was mainly distributed in the areas of bone and stained both osteoclasts and osteoblasts well. Chondrocytes were poorly stained though the chondroid matrix showed

robust staining. Younger rats had more robust staining than their older counterparts. The current study's findings of poorer staining in chondrocytes are similar to the report by Hu and colleagues (Hu et al., 2017). Osteopontin is responsible for the migration of MSCs to the fracture site (Liu et al., 2017). It is also vital in osteoclast migration and adhesion, processes key to bone remodeling (Kim et al., 2018). The current study's findings of reduced osteopontin staining in the older rats are similar to the findings of Guidi and colleagues who found reduced levels in osteoblasts and the extracellular matrix (Guidi et al., 2017). These findings confirm the pivotal role that the molecule plays in the healing of fractures.

LIMITATIONS

As chondrocytes transdifferentiate, they may appear as chondrocytes but behave as osteoblasts. A more accurate count would use specific markers of chondrocytes.

CONCLUSION

This current study has demonstrated slower healing with the use of celecoxib evidenced by lower histological grades, a lower proportion of bone, and a higher proportion of cartilage. Older animals were also more likely to have slowed fracture healing when compared with younger animals. Animals that received NSAIDs immediately after the fracture also demonstrated slower healing compared to those in which the drugs were avoided in the first week. Multivariate analysis confirmed that the combination of the use of celecoxib in the early fracture healing period in older animals resulted in the most profound slowing of the fracture healing process. The distribution of osteocalcin and osteopontin in the healing fracture callus with the use of NSAIDs was also described. The use of celecoxib in the period of inflammation in older animals was also associated with higher cell densities in the healing fracture callus. These findings indicate that the healing fracture callus in older animals given celecoxib in the early fracture healing period would be highly cellular, with low proportions of bone and persistence of cartilage even in the later stages of fracture healing. This callus is unlikely to possess the biomechanical properties required to restore the function of the limb and could lead to a delayed union. As this is an animal study, clinical studies are required to confirm these findings in humans.

RECOMMENDATIONS

Celecoxib should be avoided in the aging adult for the management of pain after a fracture. The use of NSAIDs in the first week after a fracture is discouraged and other molecules should be considered.

SUGGESTIONS FOR FURTHER STUDIES

This study has laid the ground for studies that could focus on;

a) The effect of delayed timing of NSAID administration with short and long drug term use
b) The mechanisms for altered cell densities in fracture callus after NSAID administration
c) The effect of cell densities on the biomechanical properties of bone.
d) The influence of NSAIDs on the distribution of osteocalcin and osteopontin in fracture callus

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APPENDICES

APPENDIX 1 SIGNS OF POSTOPERATIVE PAIN IN RATS

1. ‘‘Transient Stop’’- animals will be determined to have displayed this sign if, in the absence of all other ongoing activity, the animals adopt a crouched or lying posture.

2. ‘‘Twitching’’: This will be scored if the animals exhibit transient involuntary muscular contraction of any part of the body. This is usually present during the transient stop sign described above.

3. ‘‘Stagger/fall’’: This will be determined if the animals exhibit a loss of balance while walking and especially during the rapid transition to crouch from the high or low rear and/or during grooming.

APPENDIX 2 HISTOLOGICAL GRADING OF FRACTURE CALLUS

Grade 1 Fibrous tissue

Grade 2 Mostly fibrous tissue, a small amount of cartilage

Grade 3 Equal amounts of fibrous and cartilage tissue

Grade 4 Mostly cartilage, a small amount of fibrous tissue

Grade 5 Cartilage tissue

Grade 6 Mostly cartilage, a small amount of immature bone

Grade 7 Equal amounts of cartilage and immature bone tissue

Grade 8 Mostly immature bone, small amounts of cartilage tissue

Grade 9 Healing of fracture with immature bone

Grade 10 Healing of fracture with mature bone

APPENDIX 3 DATA COLLECTION SHEET

Case Number - - - - - - - - - - - - - -

Weight at Euthanasia - - - - - - - - - - - - - -

Drug Diclofenac ☐ Celecoxib ☐

Age Five Months ☐ Fifteen Months ☐

Duration of Analgesia Three weeks ☐ Four Weeks ☐

Time Point Baseline ☐ Day 21 ☐ Day 42 ☐

Histology Grade - - - - - - - - - - - - - - - -

Callus Tissue Proportions Bone - - - - - Cartilage - - - - -

Osteoblast Count - - - - - - - - - - - - - - -

Chondrocyte Count - - - - - - - - - - - - - -

APPENDIX 4 PUBLICATIONS

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Age related effects of selective and non-selective COX-2 inhibitors on bone healing

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A B S T R A C T

Introduction: Fractures are increasing worldwide and with an aging population, are frequent in the elderly. The healing of fractures progresses through various phases including the inflammatory stage. Aging is associated with slower healing and the use of non steroidal anti-inflammatory drugs (NSAIDs) may interrupt bone healing processes. We designed a study to compare the effect of diclofenac and celecoxib on fracture callus histomorphometry in a rat model of different age groups.

Methods: Using 5 and 15 month old rats, fractures were induced on the left tibia and the animals allocated to receive one of the drugs. Animals were sacrificed at day 21 and 42 and the fracture callus harvested for processing and histological evaluation. Tissue proportions and histological grades were determined and compared across the groups.

Results: Across all groups, the histological grade increased with time and animals in the young diclofenac group had the highest grade at day 42 (p ¼ 0.004). The proportion of bone increased in all groups and was highest in the young diclofenac group at day 21 and day 42 (p ¼ 0.003). Post hoc analysis showed that the young celecoxib and old celecoxib groups had the least proportion of bone (p ¼ 0.032 and p ¼ 0.003). The proportion of cartilage reduced in all groups at both time points.

Conclusion: Celecoxib was associated with lower histological grade and lower proportion of bone in older animals. We urge for caution regarding the use of celecoxib in older people for the management of pain associated with fractures. Diclofenac may be a better option in this group.

1. Introduction

Injuries including fractures are a leading cause of morbidity and mortality globally with low income countries bearing the brunt of this.[1] This has been linked to increased urbanization and mecha- nization of transport. In Africa, fractures are among the most common injuries with the limbs being the most afflicted sites.[2] The lower limb including the tibia is commonly affected. While the population of the African continent has been relatively young, it is gradually aging.[3] The high proportion of injuries and the aging population is likely to result in an increase in fractures among the elderly. The physiology of the geriatric population is different from that of young adults. In particular, the aging process has been associated with a decline in the processes of bone healing.[4,5] This is associated with a longer time to union and an increase in the risk of non union. This inevitably increases the morbidity and the cost of managing the injuries.

The process of fracture healing is known to go through several stages including hematoma formation, inflammation, fibrovascular stage, bone formation and remodeling.[6] The inflammatory stage of healing relies on the cyclo-oygenase-2 enzyme (COX-2) for the production of inflammatory markers that ultimately enable the recruitment of the necessary cells for healing.[7] Deficiency in this enzyme has been shown to result in poor bone healing.[8]

Fractures are painful conditions requiring the use of analgesics. Non steroidal anti-inflammatory drugs (NSAIDs) are commonly used in the management of limb fractures.[9] NSAIDs are also an integral component of multimodal analgesia which is important in reducing the quantity and duration of opioid use.[10] While NSAIDs are beneficial in reducing the pain and inflammation associated with a fracture, they may be detrimental to the healing of the fracture itself. The use of NSAIDs has been reported to be associated with an increased risk of non union and delayed union.[11] This effect has been thought to be related to the inhibition of the inflammatory pathway.[12]

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NSAIDs can be classified according to their inhibition of the two isoenzymes of COX with the COX-2 isoenzyme being the one responsible for the inflammatory pathway.[13] Non selective or traditional COX inhibitors including diclofenac have an almost equal effect on both isoenzymes while COX-2 selective like cele- coxib are more selective for the COX-2 isoenzyme.[14] There are suggestions that selective inhibitors of COX-2 may have a more deleterious effect when compared to non selective inhibitors.[15] Comparing a non selective with a selective COX-2 inhibitor may help to further evaluate any differences. As this effect may be even more pronounced in aged individuals, comparing young and older individuals may aid in discerning any differences.[16]

Analgesics are the most common over the counter (OTC) medication consumed by both adults and adolescents.[17,18] Both diclofenac and celecoxib are freely used and available as OTC medications in many parts of the world.[19,20] We therefore designed a study to compare the effects of diclofenac and celecoxib on fracture callus histomorphometry in the young and old using a rat model.

2. Materials and methods

Study design and population: animals

This quasi randomized study included fourty male Norwegian rats (Rattus norvegicus). Half of the animals were five months old and the rest aged 15 months. The ages of the animals were chosen to correspond to 18 and 50 human years respectively.[21] Rats were chosen as they are cheap and easy to handle. The use of the rat model has also been well established in the study of the effects of aging.[22] The sample size was calculated to detect a difference in the histological score of 0.3 at a sigma of 0.5 (a 0.05 and b 80%). In the laboratory, the animals were kept in pens floored with saw dust, at room temperature and humidity, and a 12-h light/dark cycle. They were fed on commercial pellets and given a steady supply of fresh water. Three animals in each group were used to provide the baseline data. The rest of the animals were divided into two groups of twenty animals each.

Fracture model

To simulate a common method of long bone fracture manage- ment and to standardize the fractures, a closed fracture model stabilized with an intramedullary device was chosen. At the commencement of the study, the animals were anaesthetized using ketamine 100 mg/kg and xylazine 10 mg/kg IP prior to fracture induction. The surgical area was shaved and cleaned with a prep- aration of iodine in alcohol. An intramedullary 1.25 mm Kirshner wire inserted from the knee joint into the tibia under sterile con- ditions.[23] A closed fracture of the left tibia was induced by dropping a 460 g weight from a distance of 20 cm.[24] Half the animals received
5 mg/kg of celecoxib (Pfeizer Pharmaceuticals LLC, Illertissen, Germany) while the rest received a similar dosage of diclofenac (Novartis Pharma AG, Basel Switzerland) in two divided doses, 12 h apart by oral gavage for twenty eight days.

Tissue harvesting and processing

At day 21 and day 42, five animals from each group were euthanized using 3% halothane in an airtight glass chamber until they had no pupillary reflexes and little response to pain. The an- imals were then perfused with formal saline using the transcardiac method after clearance of the blood using normal saline. The healing tibia was subsequently harvested and volume in cm3 (mls) estimated using Scherle's method.[25] The specimen was subsequently fixed in formal saline for 48 h before being subjected to decalcification in ethylenediaminetetraacetic acid (EDTA) for fourteen days with the end point determined by both the flexibility and calcium oxalate methods.[26] This was followed by dehydration in increasing concentrations of alcohol and embedding in paraffin wax before sectioning to produce ten 7 mm slides. The slides were stained using heamatoxylin and eosin (H&E) and every second slide selected for photography. Ten photomicrographs were taken for each slide using a Motic BTU8 digital camera (Motic, Kowloon, Hong Kong) mounted on a Ritchter Optica UT1 microscope (Ritchter Optica, Kowloon, Hong Kong). Every second image was used for histological analysis and stereology using and Image Jsoftware (NIH, Massachusetts, USA).

Statistical methods

The histological grade was determined using the ten point scale by Huo et al.[27] in which 1 represents mesenchymal fibrous tissue and 10 is mature bone. The volume density of bone, cartilage and fibrous tissue was determined by counting the points falling on a specific tissue and dividing by the number of points falling on the fracture callus. The volume density was determined using Vv_{tis} ¼ Pt_{tis}/ Pt_{tot}*100, where Pt_{tis} is the number of points fall- ing on the tissue; Pt_{tot} is the total number of points falling on the fracture callus and Vv_{tis} is the volume density of the tissue (Fig. 1). Bone was described as vascularized tissue with cells in an osteoid matrix, cartilage as rounded cells in lacuna in a hyaline matrix while fibrous tissue as spindle-shaped cells in a fibrous matrix.

The data from the Image J was extracted into Ms Excel

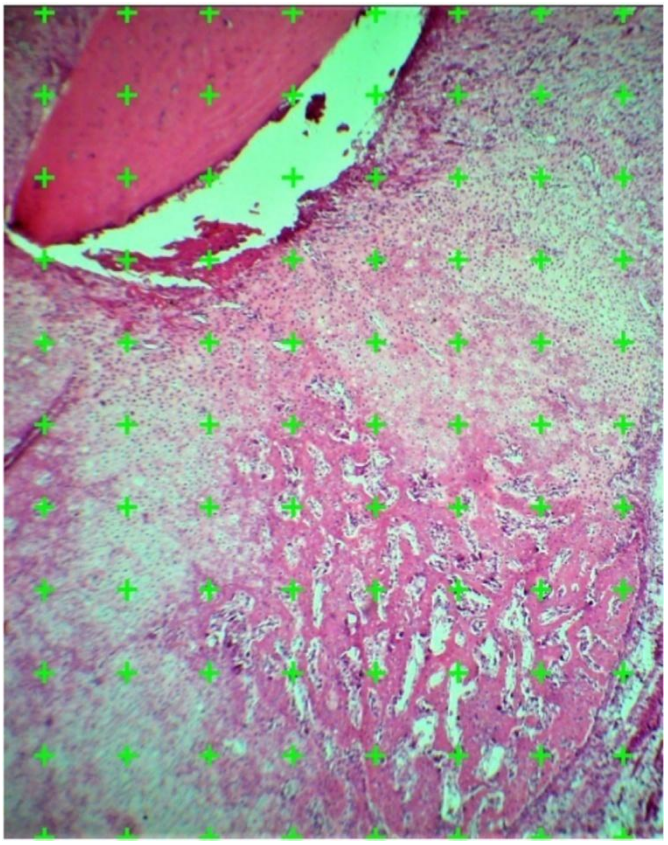

Fig. 1. Histological micrographs showing a section of fracture callus with a super-imposed set of points for estimating volume densities generated using the Image J software.

(Microsoft Corp, Washington, USA) before exporting to SPSS v17.0 (SPSS Inc, Chicago, USA) for analysis. After determining normality of the data by histograms and box plots, the means, medians, standard deviations and interquartile ranges were calculated as appropriate. The one way ANOVA and Kruskal Wallis tests was used to compare the means and medians across the four groups and the p was set at 0.05. Post hoc analysis was performed using Tukey HSD and Dunn tests respectively.

Ethical considerations

Ethical approval was obtained from the animal ethics committee of the University of Nairobi and the animals were handled in accordance with the Home Office Guidance on the operation of the Animals (Scientific Procedures®) Act 1986, published by HMSO, London (or the comparable guidelines for the state of Israel). All procedures were performed according to the Guide for Care and Use of Laboratory Animals (NIH publication No. 85e23, revised 1985).

3. Results

A total of 40 male rats were included. At day 21 the diclofenac group (A) had more bone as compared to the celecoxib group whichwas mainly cartilage (B). At day 42, the diclofenac group (C) had a higher proportion of bone while the celecoxib group had a mixture of bone and cartilage (D) (Fig. 2).

The histological grade of all groups increased with time but was higher in the diclofenac group in both age groups (Fig. 3). While at day 21 there was no statistically significant difference in the histological grade (p ¼ 0.299), by day 42 the difference across the

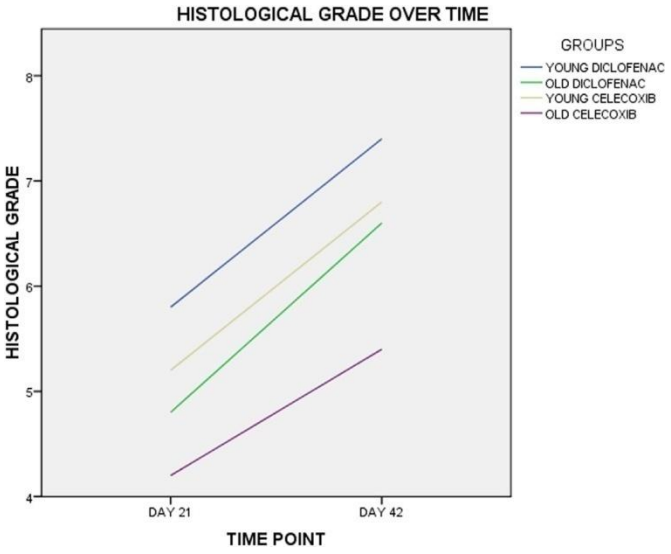

Fig. 3. Histological grade.

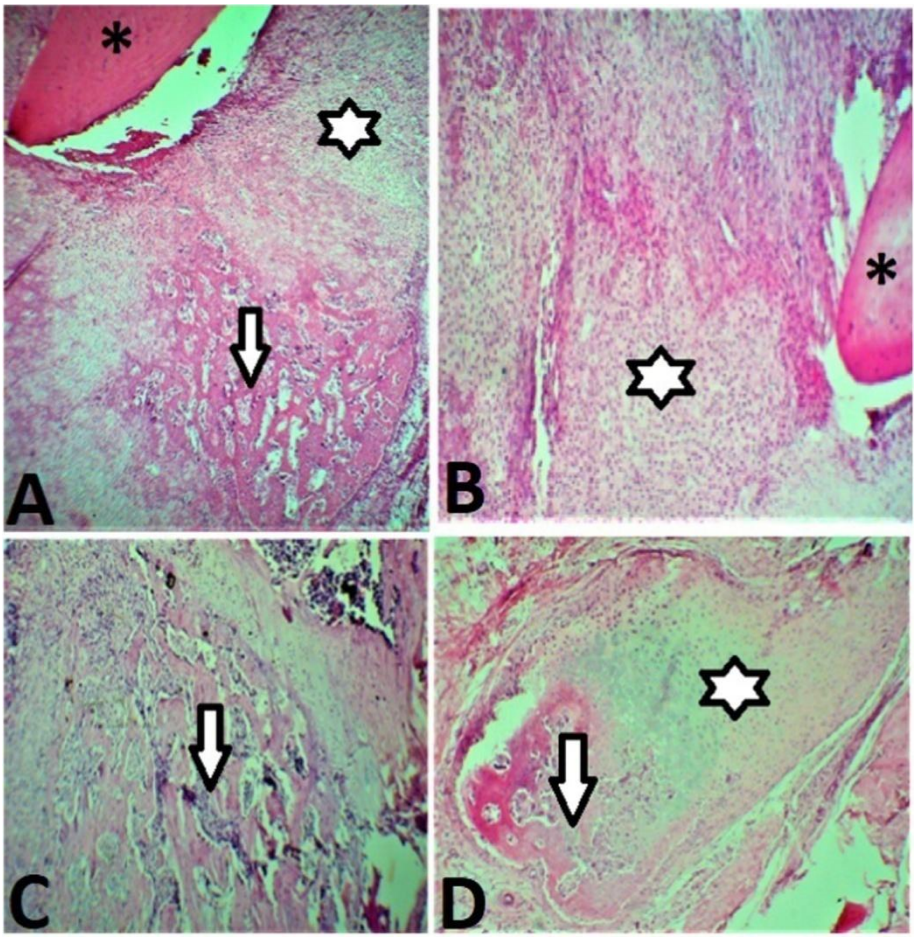

Fig. 2. (AeD). Photomicrographs of the fracture callus showing the fractured end of bone (asterix), the area of new bone (arrow) and the areas with cartilage (stars); Hematoxylin and Eosin stain X40. Figures A and C are the young diclofenac groups at day 21 and 42. Figures B and D are the old celecoxib groups at day 21 and 42.

groups was statistically significant (p 0.004) and post-hoc anal- ysis revealed that the difference was between the young animals that received diclofenac and the old animals that received celecoxib at day 42 (p 0.002).

The proportion of bone in the fracture callus increased in all groups over time and the differences were statistically significant at day 21 (p 0.003) and day 42 (p 0.003). Post hoc analysis revealed that at day 21, the proportion of bone was higher in the young diclofenac group when compared to the young celecoxib and old celecoxib groups (p 0.032 and p 0.003 respectively). At day 42 the young diclofenac group had a higher proportion of bone than the young celecoxib and old celecoxib groups (p 0.045 and p 0.003 respectively).

The proportion of cartilage in the fracture callus reduced in all groups over time (Fig. 4). Animals that received celecoxib had higher proportions of cartilage that those that received diclofenac but this difference was not statistically significant at day 21 and 42 (p ¼ 0.222 and p ¼ 0.056).

4. Discussion

The present study shows that the proportion of bone in the fracture callus increased over time in all the studied groups. This was more pronounced in younger animals and those that received diclofenac. The proportion of cartilage was higher in the animals that received celecoxib. The process of fracture healing is known to go through several stages including hematoma formation, inflam- mation, fibrovascular stage, bone formation and remodeling.[6] The bone is formed mainly by endochondral ossification in which cartilage formation precedes bone. It is thus expected that the proportion of cartilage should reduce while that of bone increases as the fractures heal and the findings of this study are similar to others in literature.[28]

A new finding in this study is the demonstration that increasing age and use of COX-2 selective inhibitors resulted in persistence of cartilage and lower proportions of bone in the fracture callus. While celecoxib has been shown to inhibit the chondrogenic phase of fracture healing,[28] this study reveals that this effect is worse in older age groups. Older animals have been shown to have reduced fracture healing potential which may be related to senescence of the osteoprogenitor cells.[29,30] The differences in proportion of bone between the young and old animals that received a particular NSAID were not statistically significantly different. Conversely, statistically significant differences were seen when we compared the young diclofenac group with the old celecoxib group. This implies that the differences seen cannot be attributed to age alone and the drug used was a factor as well. This study therefore dem- onstrates that older animals may suffer the deleterious effects of COX-2 inhibitors more that younger ones. The findings in this study could be due to celecoxib inhibiting the COX-2 prostaglandin 2 (PGE2) pathway that mesenchymal stem cells (MSCs) rely on to promote osteogenesis in the initial stages of fracture healing.[31] As the selective COX-2 inhibitors preferentially inhibit this enzyme, it is likely that their effects are more profound when compared to the non-selective NSIADs.

At a molecular level, this could be due to celecoxib suppressing Wnt target genes resulting in reduced expression of runt-related transcription factor 2 (RUNX2) and ALP consequently inhibiting osteoblast-mediated mineralization.[32] Celecoxib has also been implicated to reduce the expression of Receptor activator of nuclear factor-kB ligand (RANKL) providing another probable mechanism of action.[33] The consequence of this is reduction in the mechanical properties of the fracture callus, delayed union and non union. While NSAIDs are important agents in the management of pain after fractures, this study highlights the need to avoid the use of selective COX-2 inhibitors in the elderly. Although some non se- lective NSAIDs may have lower COX 2 selectivity, the choice of diclofenac in this study was informed by the most common mol- ecules in clinical use.

To manage pain associated with fractures or their surgical correction, several options are available to the surgeon including paracetamol (acetaminophen), NSAIDs and opiods. This study demonstrates that selective COX-2 inhibitors may be unsuitable in the elderly to manage this pain as this may lead to delayed and non union. The surgeon should use other molecules available in his armamentum.

Limitations

While this study demonstrates differences in the histological structure of the fracture callus, evaluation of the healing callus mechanically and radiologically may provide greater insights into the potential clinical implications of our findings. The choice of drugs in the study was informed by the most common drugs in each of the classes; evaluation of more molecules may help eluci- date if the findings seen are a class effect or are specific to indi- vidual molecules.

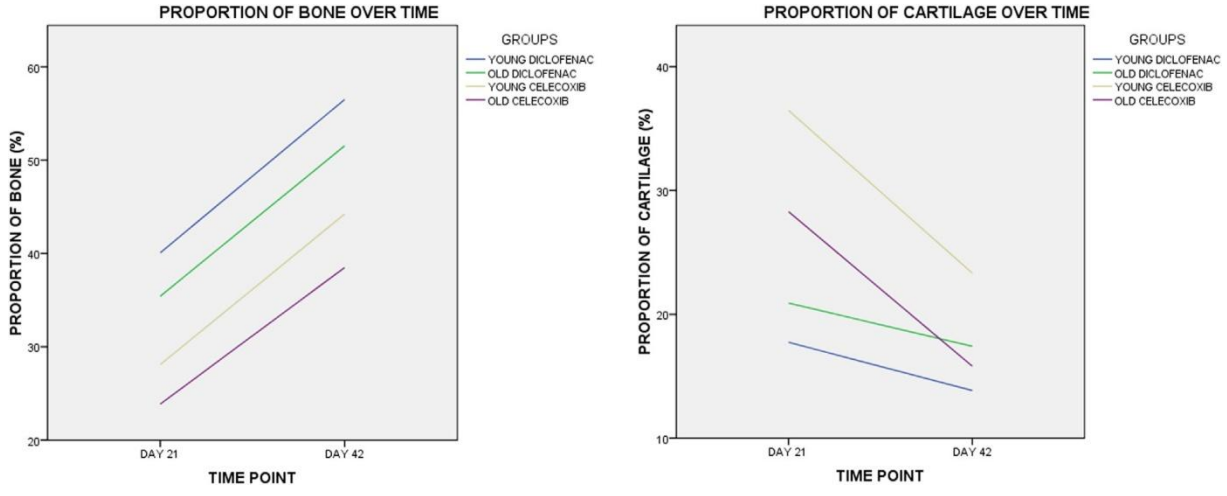

Fig. 4. A and B. Proportion of bone and cartilage in the fracture callus.

5. Conclusion

This study demonstrates poor fracture healing in older age groups with the use of celecoxib manifest by lower histological grade and a smaller proportion of bone. This could result in a me- chanically weaker union and delayed union. Use of selective COX-2 inhibitors in the elderly in the setting of fractures should be discouraged. Further studies to quantify the inhibition pathways forbone healing between the young and old rats are warranted.

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Declaration of competing interest

The authors have no conflicts to declare.

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EFFECT OF TIMING OF COX-2-SELECTIVE NSAID USE ON FRACTURE HEALING IN AN ADULT RAT MODEL

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ABSTRACT

Introduction: Fractures are common in the elderly and are associated with increased morbidity. The pain of fractures and surgery can be managed using NSAIDs, but this may result in impaired healing. The inflammatory stage of bone healing is responsible for laying the foundation for subsequent proliferative stages. This may be the stage when NSAIDs may have their greatest impact and it is unclear if avoiding NSAIDs in this stage would result in differences in healing or whether different molecules have varied responses. This study sought to determine the differences in the histomorphometry of fracture callus in older rats when diclofenac and celecoxib were avoided in the first week after a fracture.

Methods: Fractures of the tibia were induced in 40 15-month-old (equivalent to 50 human years) rats which were then allocated to receive either diclofenac or celecoxib. Each group was further subdivided into early or late subgroups of 10 animals each receiving the study medication from the day after the fracture or eight days later, respectively. Histological and stereological examination of the callus at days 21 and 42 enabled comparison of histological grades, tissue proportions and cellular density.

Results: The histological grade and amount of bone increased and the cartilage reduced in all groups. The group that received celecoxib early had the least proportion of bone. The osteocyte and chondrocyte cellular densities increased in all groups across both time points.

Conclusion: Administration of celecoxib in the early fracture period in the old is associated with poorer histological scores, lower proportions of bone and increased cellularity which may result in

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delayed union of the fracture. Use of selective COX-2 inhibitors is discouraged for the management of pain in older patients with fractures especially in the first seven days.

Keywords:

INTRODUCTION

Fractures are common in the older age groups and are associated with high morbidity and increasing mortality.[8] An older adult is at an increased risk of delayed union because of a diminution of the healing process that increases with age.[3] This may further worsen the outcomes of the management of these injuries. Fractures and surgical procedures to manage them are painful and non-steroidal anti-inflammatory drugs (NSAIDs) are useful in reducing this pain.[7] While NSAIDs will relieve pain, they have also been associated with impaired fracture healing because of their influence on the cyclo-oxygenase-2 (COX-2) enzyme.[5]

Fracture healing goes through several stages beginning with the inflammatory stage which is important as it lays the foundation for the recruitment of the cells and factors necessary for healing.[17] The inflammatory stage of fracture healing is estimated to last about a week and gives way to the more proliferative fibrovascular and bone forming stages.[12] It is likely that any effect of NSAIDs on fracture healing occurs during the inflammatory stage by inhibiting the COX-2 enzyme which would then influence the subsequent stages.

Based on their inhibition of the COX-2 enzyme, NSAIDs can be classified into nonselective inhibitors including diclofenac and selective inhibitors like celecoxib.[2] While both categories have been shown to affect fracture healing, the latter category of selective inhibitors has been associated with more derangement of the fracture healing process.[5] Both short-term and prolonged uses of celecoxib have been associated with impaired fracture healing in rats.[11] It is, however, unclear what role, if any, commencing drug administration at different timings would have in the pathway to the effect of these molecules. This would require avoiding NSAIDs in the first seven days when the fracture is in the inflammatory stage to determine if this may result in less disruption of the healing process. The combination of reduced healing potential due to senescence and the effect of NSAIDs may result in high incidences of delayed union and nonunion in the elderly, while the effects may not have manifested in the young. This study sought to determine the effects on fracture callus histology after avoiding NSAIDs in the first week after a fracture in an elderly rat model.

MATERIALS AND METHODS

Experimental Model

In this quasi-experimental study, 43 15-month-old male rats (*Rattus norvegicus*) were divided into two groups; one group to receive 5 mg/kg of diclofenac (Novartis Pharma AG, Basel, Switzerland) and the other group a similar dosage of celecoxib (Pfizer Pharmaceuticals LLC, Illertissen, Germany) by oral gavage in two divided doses daily. Three animals were used to determine the baseline morphology and the rest were divided so that one half received the study medication from the first day and the other half received the medication from the eighth day until day 28 (Fig. 1). Anesthesia was achieved using intraperitoneal (IP) ketamine (100 mg/kg) and xylazine (10 mg/kg). A weight (460 g) was dropped from a height (20 cm) resulting in a closed transverse fracture of the mid-tibia.[15] Animals with open fractures and injuries other than the mid-tibia fracture were excluded.

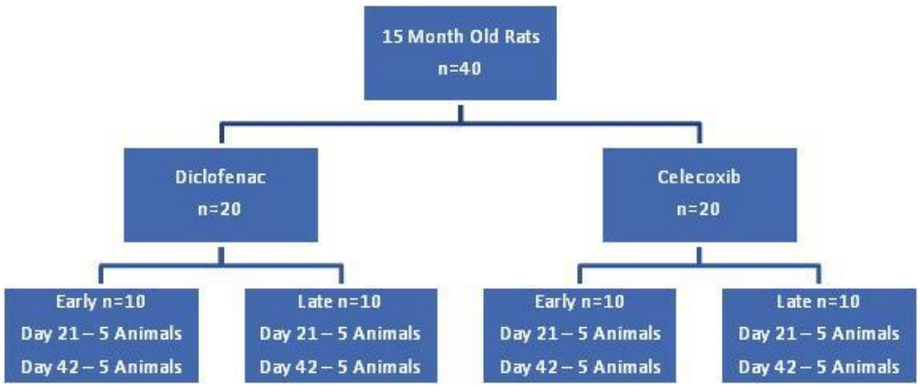

Fig. 1 Distribution of the experimental animals.

Ethics

Ethical clearance from the Institutional Biosafety and Animal Use Ethics Committee was obtained prior to the commencement of the work and the animals were selected according to the Guide for Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1985).

Histology and Stereology

Baseline morphology of the fracture callus was determined by sacrificing three animals at the seventh day after fracture, while five animals from each of the four subgroups were sacrificed at days 21 and 42. The healing tibia was dissected of the animals and subjected to fixation in formal saline before decalcification. Dehydration in increasing alcohol concentrations was performed prior to embedding in paraffin wax and sectioning 7-∝m slides. Five randomly selected slides per animal were used for microscopic examination, where five areas were selected for photography using a Sony Cyber-shot [12.0 MP (megapixels)] digital camera. Image analysis was performed using Image J software (NIH, MA, USA) and processed using Excel (Microsoft Corporation, Redmond, USA).

To determine the volume density of bone and cartilage, the points on each tissue were divided by the points on the whole fracture callus at 100× magnification.[9] The numerical density of osteocytes and chondrocytes was determined by counting the number of cells using a count-ing frame with a forbidden line at 400× magni-fication (Fig. 2). The sampling at the image level was done by starting at a fixed point at the top-left-hand corner and moving with a constant step towards the right. The numerical density for each slide was determined using the formula:

$Nn_{cell} = \sum Q\text{-}_{cell}/\sum A_{tot}$, where $Q\text{-}_{cell}$ was the num-ber of cells counted and A_{tot} was the area of the counting frame. The average for each animal was determined.

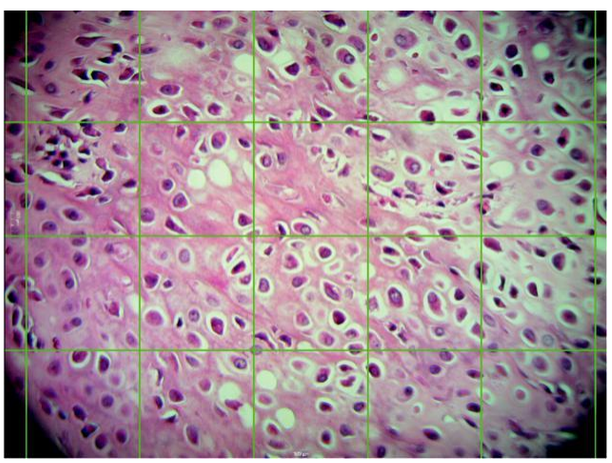

Fig. 2 Histological micrograph with a superimposed grid for estimating the numerical densities of the cells: Hematoxylin and Eosin stain 400×.

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Statistical Methods

The sample size could determine differences in the histological score as small as 0.3 at the α of 0.05 and β of 80%. Data from the spreadsheet were analyzed using SPSS v17.0 (SPSS Inc., Chicago, IL, USA). After ascertaining the normal distribu-tion, means and standard deviations were calcu-lated and the one-way ANOVA was used to test the statistical significance with the *p*-value set at <0.05. Tukey's HSD test was carried out in the post-hoc analysis.

RESULTS

There was an increase in the histological grade in all groups from day 21 to day 42 (Fig. 3) with the difference at day 42 rising to statistical signif-icance (p = 0.036). This difference was attributed to the diclofenac late and celecoxib early groups (p = 0.029).

Bone tissue increased in all groups with a sta-tistically significant difference noted at day 42 (p = 0.049) attributable to the difference between diclofenac late and celecoxib early (p = 0.045). While the levels of cartilage reduced over time in all groups and remained higher in the celecoxib early group, this was not statistically significant (Table 1).

At day 21, the group that received diclofenac late [Fig. 4(a)] had almost equal bone and carti-lage, while the early celecoxib group had a high proportion of fibrous tissue [Fig. 4(c)]. While at day 42, the late diclofenac group had mainly bone

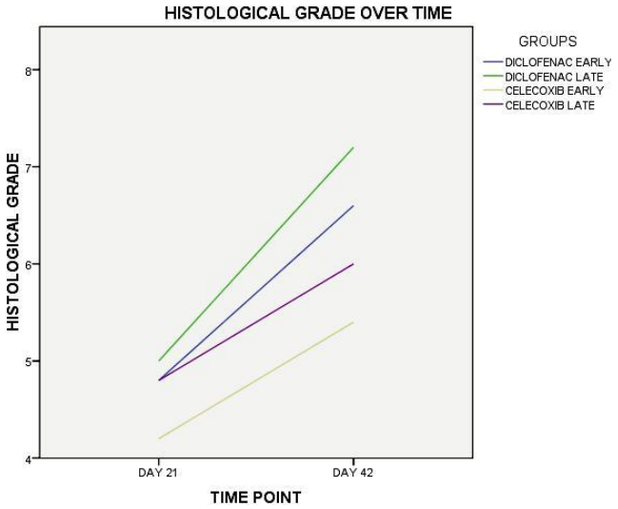

Fig. 3 Histological grade over time.

with little islands of cartilage [Fig. 4(b)], whereas the early celecoxib group had a high proportion of fibrous tissue [Fig. 4(d)].

Both the osteocyte and chondrocyte numer-ical densities increased in all groups across day 21 and day 42. The differences in the osteocyte numerical density were not statistically signifi-cant at day 21. At day 42, the differences were statistically significant and post-hoc analysis revealed that the differences were between diclofenac early and celecoxib early (p = 0.007), between diclofenac late and celecoxib early (p = 0.011) and between celecoxib early and celecoxib late (p = 0.004). The chondrocyte numerical density did not show any statisti-cally significant differences at both time points (Table 2).

Table 1 Proportion of Various Tissues in the Fracture Callus.

		Proportion of Bone % (SD)			Proportion of Cartilage % (SD)		
		Early	**Late**	***p*-Value**	**Early**	**Late**	***p*-Value**
Day 21	Diclofenac	35.42 (6.20)	36.65 (6.32)	0.123	20.90 (13.44)	38.33 (14.17)	0.278
	Celecoxib	23.85 (3.77)	33.71 (4.50)		28.29 (15.24)	28.97 (17.24)	
Day 42	Diclofenac	51.53 (4.68)	54.25 (2.92)	0.049	17.41 (1.07)	20.99 (5.92)	0.497
	Celecoxib	38.49 (9.34)	46.46 (13.24)		15.80 (4.78)	15.41 (9.83)	

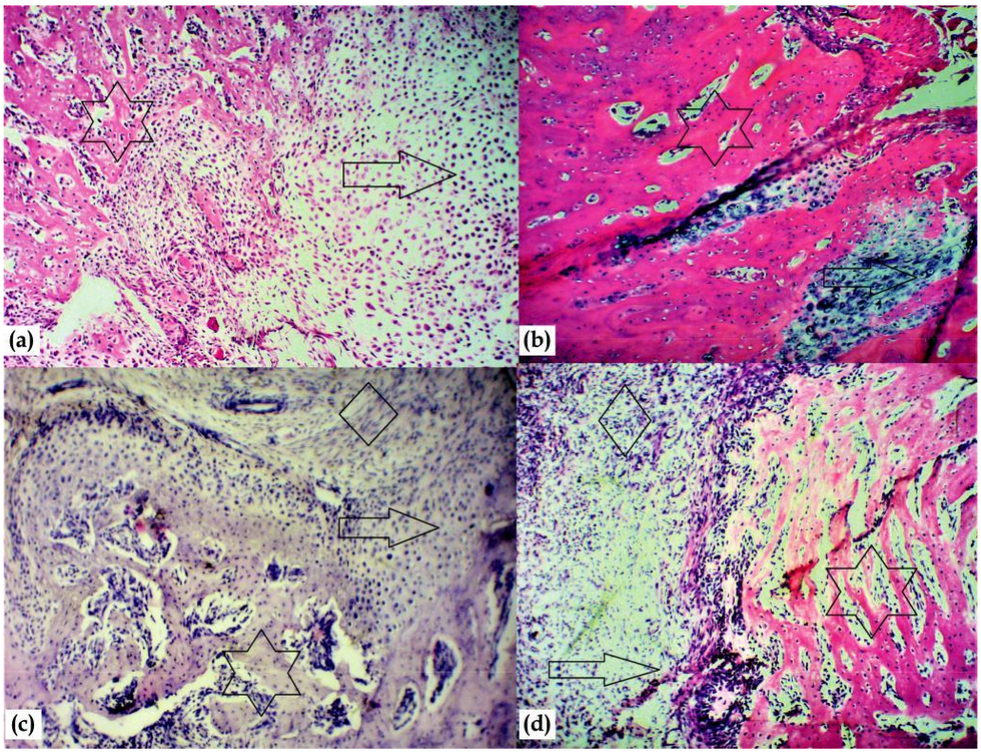

Fig. 4 **(a)–(d)** Photomicrographs of the fracture callus showing areas of bone (stars), cartilage (arrow) and fibrous (diamond): Hematoxylin and Eosin stain 100×. Panels **(a)** and **(c)** are at day 21, while panels **(b)** and **(d)** are at day 42 (diclofenac late and celecoxib early, respectively).

Table 2 Cellular Numerical Densities.

		Osteocyte Density (/mm^2) (SD)			Chondrocyte Density (/mm^2) (SD)		
		Early	**Late**	***p*-Value**	**Early**	**Late**	***p*-Value**
Day 21	Diclofenac	85,300 (33,506.30)	93,376.19 (27,229.93)	0.303	134,609.52 (46,799.64)	213,828.57 (34,861.73)	0.217
	Celecoxib	112,600 (8532.29)	94,278.57 (9096.25)		134,600 (117,101.13)	177,692.64 (24,706.96)	
Day 42	Diclofenac	104,000 (11,464.24)	106,930 (31,091.62)	0.002	173,300 (64,011.32)	215,383.33 (8472.09)	0.068
	Celecoxib	157,130 (21,608.38)	100,820 (18,549.86)		149,333.33 (43,852.65)	209,142.86 (24,940.74)	

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At day 42, the celecoxib early group had areas of bone that were highly cellular with numerous vascular channels. The cells were plump indicative of high metabolic activity [Fig. 5(a)]. The diclofenac late group had mainly mature bone with lower cellularity. The cells were smaller depicting a more quiescent stage while the matrix was more regular in orientation [Fig. 5(b)].

DISCUSSION

There was an increase in the proportion of bone and the histological grade across all groups and

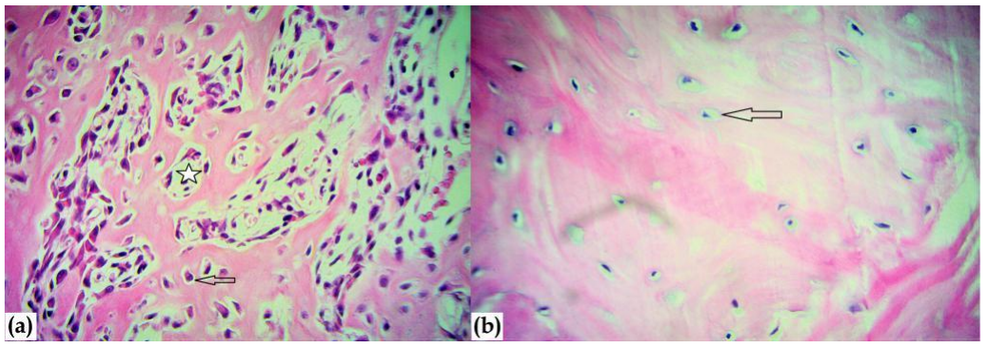

Fig. 5 **(a)**, **(b)** Photomicrographs highlighting osteocytes (arrows) and vascular channels (star): Hematoxylin and Eosin stain 400×. Panels **(a)** and **(b)** are the early celecoxib and late diclofenac groups at day 42, respectively.

the difference between the diclofenac late and celecoxib early groups was statistically signif-icant. Bone healing advances in several stages resulting in the progressive replacement of the fibrovascular tissue with cartilage and even-tually with bone.[17] It is thus expected that the proportion of bone should increase as that of car-tilage falls. A striking result in this study is the demonstration of differences in the histological grade based on the timing of administration of the study medication. The group that received celecoxib in the inflammatory stage had statis-tically significantly reduced histological scores. Post-hoc analysis did not show statistically sig-nificant differences either between celecoxib and diclofenac or between early and late. Hence, the difference seen is as a result of the combina-tion of celecoxib and its early administration in the elderly. Celecoxib has long been associated with inhibition of bone formation and this has been attributed to its inhibition of various mol-ecules.[13,1,6] Though this inhibition may be caused by all NSAIDs, it is likely that this is greater with celecoxib than diclofenac. Similar to our find-ings, a previous review concluded that NSAIDs administered in the first seven days resulted in poorer bone healing.[10] This study, however, pro-gresses that discussion by comparing two dif-ferent drugs and finding that selective COX-2 inhibitors represented by celecoxib may cause a more profound disruption of healing than their nonselective colleagues when administered early in the older individuals.

While the levels of bone increased and car-tilage reduced over time in all groups, the cele-coxib early group had statistically significant lower proportion of bone when compared to the diclofenac late group. This reinforces the earlier finding of a poorer histological score and points to the possibility of poorer bone healing when celecoxib is administered early in the older ani-mals. The combination of a poorer histological score, a lower proportion of bone and a higher proportion of cartilage demonstrates that admin-istering celecoxib in the early fracture period in the elderly results in poorer healing of fractures. As these findings were not seen when the drugs were compared controlling for timing or when the timing was compared controlling for drug, the effects seen are as a result of the combination of celecoxib, its administration in the early frac-ture period and the advanced age of the animals. The aging process results in slower and poorer healing of bone which results in delays or failure in the union of the fracture.[4] It is therefore likely that the elderly will be more susceptible to any further insults to the fracture healing processes as celecoxib has been shown to do. Fracture

healing progresses through various stages from the formation of a hematoma, cellular proliferation to the progressive replacement of cartilage by bone.[12] The formation of bone is the hallmark of healing and restores the structural integrity of bone. A delay or failure to achieve this, as seen in the celecoxib early group, results in delayed- and non-union of the fracture. While we have previously demonstrated that older animals receiving celecoxib have a reduced fracture healing potential, this study progresses the discussion by finding that the timing of the drug may hold the key to its effects.[9] This introduces a "window period" where the use of the drug may need to be avoided if there are no viable analgesic alternatives.

There was an increase in the density of osteocytes and chondrocytes in all groups over time. This study also noted that at day 42 the osteocyte density in the celecoxib early group was higher than the other groups. The density of the osteocytes and chondrocytes is expected to increase, plateau and later fall as the fracture callus matures.[16] The new finding in this study is the increase in cellular density in the group that received celecoxib early compared to those that received the drug late or received diclofenac. NSAIDs have been shown to affect the biomechanical strength of bone and this is likely due to the effect on the fracture callus extra cellular matrix (ECM).[11] It is likely that the group that received celecoxib early had reduced biomechanical properties which resulted in the tissue reacting by increased proliferation of cells. This should be the focus of future studies.

While small animals especially rats have been extensively used for basic science research of fractures especially to expound on the inflammatory and regenerative processes, the structure of rodent bone is more primitive that that of humans.[14] Our findings, even though not directly translated to humans, provide insight on the possible effects of NSAIDs on fracture healing. In total, these findings point to a likelihood of poorer healing when celecoxib is administered in the early fracture healing period in older individuals- . This could result in delayed- and non-union of the fracture.

Limitations

While this study has demonstrated various changes in the histomorphology of the fracture callus, biomechanical studies could have added much needed information. Although diclofenac is known to have weak COX-2 inhibition, it selection was based on the common molecules used in orthopedic clinical settings. The duration of drug administration may have influenced the results and future studies may evaluate the timing while ensuring equal durations of drug use.

CONCLUSION

This study demonstrates that celecoxib adversely affects fracture healing when administered in the first week after a fracture in older animals. This is evidenced by poorer histological scores, less bone formation and increased bone cellularity. As this could result in delayed healing, we advocate that clinicians avoid the use of COX-2-selective NSAIDs including celecoxib after a fracture in older individuals. If these molecules need to be used, they should be avoided in the first week after a fracture.

AUTHOR CONTRIBUTIONS

James W. M. Kigera did the conceptualization, methodology design, investigation and writing of the original draft. Peter B. Gichangi, Adel K. M. Abdelmalek and Julius A Ogeng'o were involved in the conceptualization, writing — review and editing — as well as supervision.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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