The Detrimental Effects of Cigarette Smoke on Fracture Healing in a Bilateral Femur Fracture Rodent Model

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THE DETRIMENTAL EFFECTS OF CIGARETTE SMOKE ON FRACTURE HEALING IN A BILATERAL FEMUR FRACTURE RODENT MODEL

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
Dr. Russell A. Reeves, MD
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ABSTRACT:

Fractures that fail to heal (nonunions) substantially contribute to patient morbidity and overall healthcare costs. When nonunions occur in the absence of a critical size defect it is the result of impaired healing via one of the two distinct bone healing pathways: primary (intramembranous) and secondary (endochondral) ossification. Furthermore, the recent characterization of circulating osteoprogenitor cells (COPs) presents yet another important mechanism by which fractures may heal. Clinically, patients who smoke cigarettes are at increased risk of suffering from a nonunion; however, the mechanism by which this occurs has still not yet been defined. We sought to determine whether cigarette smoking exerts a differential effect on two distinct bone healing pathways with an aim to define the optimal treatment approach for patients who are active smokers and to explore whether smoking cigarettes affects the production of COPs. To do this, we aimed to (1) Establish a reproducible smoke-exposure protocol for use in vivo with the Sprague-Dawley rat, (2) Develop a bilateral femur fracture model for measuring the effects of cigarette smoke on both primary and secondary healing pathways, (3) Determine whether cigarette smoke exerts a preferential inhibitory effect on cartilage production in the endochondral ossification pathway and (4) Measure the production of COPs and compare their induction under the condition of smoke exposure.

Forty-six male Sprague-Dawley rats were obtained, half (n = 23) were exposed to daily one-hour cigarette smoke exposures, 12 sessions per week, for one month preoperatively and one month postoperatively. A goal of 200mg/m³ total particulate matter (TPM) was set for each smoke exposure. All animals received bilateral femur
fracture surgery, one side treated with an intramedullary nail and the opposite treated with a compression plating technique. COPs were measured by identifying CD34, osteocalcin double positive cells via flow cytometry. Animals were harvested at 10 days, 1 month, 3 months and 6 months postoperatively. Endochondral ossification was assessed using Safranin-O histomorphometry and microCT assessment of calcified callus volume. Fracture healing strength was assessed via 4-point bending at 3 months and 6 months postoperatively. Statistical analyses were performed using two-way ANOVA with Fisher’s least significant difference for post-hoc comparisons. A p-value less than 0.050 was considered statistically significant.

Smoke-exposed animals received an average an average TPM exposure of 200.6 ± 73.0 mg/m³ (95% CI: 193.9 - 207.3, Range: 61 - 704), without any difference in average exposures between animals (one-way ANOVA, p = 0.061). All animals exhibited fracture healing postoperatively via plain X-ray radiographs without apparent impairment in gait. Animals lost a significant amount of bodyweight after 10 days of cigarette smoke exposure (mean difference: 50.1, 95% CI: 19.4, 80.8, p = 0.002) and this difference was maintained following one month of smoking cessation (mean difference: 50.2, 95% CI: 26.3, 74.2; p <0.001). This was accompanied by a corresponding decrease in food consumption in the smoke-exposed animals (smoking day 10, mean difference: -6.56, 95% CI: -9.54, -3.59; p < 0.001); however, no differences were seen in plasma transthyretin levels, a marker of nutrition. At 10-days postoperatively, cartilage formation in the nailed femurs was inhibited in response to smoke exposure [Mean difference (control plate vs control nail): 0.222mm², 95% CI (0.023, 0.420), p = 0.033]. COP
mobilization was decreased in response to cigarette smoke at 4 days and two weeks following fracture surgery (p ≤ 0.047). At four weeks following surgery, smoke-exposed animals demonstrated increased COP mobilization compared to controls (p ≤ 0.041).

This study represents the successful establishment of a smoke-exposure protocol in a bilateral femur fracture rodent model. Smoke exposure exerted an anorexigenic effect and subsequent weight loss; however nutritional impairment was not evident. Endochondral ossification was substantially impaired as evidenced by the reduced cartilage production in the nailed specimen. Early COP production was also found to be impaired in response to smoke exposure, but enhanced at four weeks. These results offer a unique understanding of how cigarette smoke plays a role in fracture biology, specifically in its inhibitory effect on secondary bone healing and on early COP recruitment. These results imply that choice of the optimal fracture fixation method may be dictated by the differential effects of smoking on the two fracture healing pathways. Future work will investigate the effects of smoke exposure on production of the calcified callus and acquisition of mechanical strength through each of the fracture healing pathways.
DEDICATION

This work is dedicated to my grandfather, who shares my first name. Both a self-starter and industrious, his dedication taught me that nearly anything can be accomplished through persistence. His hard-work and generosity toward others continues to inspire me in nearly every endeavor.
ACKNOWLEDGEMENTS

This work would not be possible without the mentoring and support from Dr. Vincent D. Pellegrini, Jr., MD. His guidance throughout this study has been key toward gaining a better understanding of the pathophysiology of fracture-nonunions. Furthermore, he has allowed me to take initiative with this study, giving me the chance to develop both personally and professionally from the responsibilities of managing an active laboratory. I’d also like to acknowledge Dr. Yongren Wu, PhD, an instrumental collaborator on all aspects of this research. Yongren was a key assistant in all fracture surgeries throughout this study and has coordinated nearly all sample processing. I am truly inspired by Dr. Wu’s work-ethic and ability to perform under stress. His both professional and amical nature made completion of this project both satisfying and enjoyable. I aspire to someday share Dr. Wu’s mild manners and ability to focus. A great deal of this work would not have been possible hadn’t it been for the efforts of Glenn Hefter, who had contributed as both anesthesiologist but also as an all-around team player. A number of medical students helped throughout this process, naming Elizabeth Nadeau, Sarah Guess and Tucker Kornegay, to name a few.

In terms of collaborators, Dr. Amanda LaRue, PhD and Dr. Ryan Kelly, PhD have been crucial collaborators throughout this investigation. Both have helped me immensely in studying the effects of cigarette smoke on the production of circulating osteoprogenitor cells. Furthermore, several assays would not have been possible hadn’t it been for their resources. Dr. Tong Ye, PhD and Yang Li have spent countless hours helping me study the effects of cigarette smoke on chondrocyte viability in response to cigarette smoke
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CHAPTER ONE
INTRODUCTION

Background Clinical Relevance

Smoking cigarettes produces numerous deleterious effects on the human body
[16, 17, 75]. Approximately 11.4% of the US population smokes and as such, it is vitally
important to understand the mechanisms by which cigarette smoke imparts its negative
effects on the human body [18, 47, 70, 85]. Despite the wealth of research on the effects
of cigarette smoke on cardiac, pulmonary, metastatic and vascular diseases [16, 17, 37,
87], relatively few mechanistic studies have explored the effects of cigarette smoke on
the functions of the appendicular skeleton [32, 75], particularly the biology of fracture
healing [81]. Cigarette smoking is the fourth most important risk factor in the
development of fracture nonunions and is the most important modifiable risk factor
identified thus far [82]. Given that fracture nonunions are common [98] and contribute to
significant patient morbidity, the need for additional procedures and increased overall
costs [35], gaining a better mechanistic understanding of why these complications occur
is essential.

There is a wealth of information that associates cigarette smoking to poor
perioperative outcomes in orthopaedic patients; patients who smoke demonstrate both
reduced and slower fusion rates following arthrodesis (joint fusion) procedures and also
have increased risks of nonunion following fracture fixation [1, 54, 75]. Despite these
associations, the mechanistic etiology of fracture nonunions in smokers is less clear
because of the limited basic science research in this area. The laboratory study of fracture
healing has been limited for several reasons: (1) performing reproducible smoke-exposure in laboratory animals is technically challenging, (2) high costs and resources are required to provide reproducible exposures and (3) the establishment of fracture-biology animal models which are not only reproducible, but also relevant to orthopaedic surgical patients has been limited. Cigarette smoke exposures involve the design of intricate equipment that is able to reliably load, ignite and generate cigarette smoke. The quality, concentration and volume of smoke exposure must be tightly controlled and maintenance of this equipment is time consuming and costly. As such, few investigators have studied the influence of smoke-exposure itself on fracture healing biology [56, 81], with the majority of investigators using administration of nicotine [20, 22, 33, 36, 41, 62, 77, 96] or tobacco extract [29, 34, 86] as surrogates for smoke exposure. The act of burning cigarettes produces over 500 gases and the particulate (solid phase) contains over 3,500 different chemical substances, which include nicotine, but also many other toxic compounds such as cyanide and carbon monoxide [40]. Although the link between nicotine and bone cell function, osteoneogenesis and bone turnover is well established [50], the link between smoking and fracture healing is less clear, as several studies have shown that impaired fracture healing could be due to toxic compounds other than nicotine [29, 34].

The biologic process of fracture healing is complex and occurs through two main pathways: primary bone healing via intramembranous ossification and secondary bone healing through endochondral ossification. The distinction between these two healing pathways is through the development of a large cartilaginous callus intermediate in
endochondral ossification, which forms an initial cartilaginous scaffold that later becomes calcified and permits bony callus formation. The bony callus that is formed through endochondral ossification persists for months to years after the initial fracture and eventually remodels in response to the stresses and strains of weight-bearing according to Wolff’s Law. The stimulation of endochondral ossification occurs in response to the relative instability within fracture segments and acts as a provisional stabilizer in order to allow the bone to heal. In contrast, intramembranous ossification occurs when fracture segments are rigidly fixed to one another and compressed end-to-end. This type of rigid fixation mitigates the need for provisional stabilization through a fracture callus and as such, bone healing occurs without the formation of a callus and by means of the same mechanisms as bony remodeling [26]. Given that these two pathways occur based on the relative stability of fracture segments, the surgical fixation method determines the pathway by which a bone heals. Orthopaedic surgeons regularly use fixation devices that offer either relative or absolute stability, two examples being intramedullary nailing or compression plate fixation, respectively. Choice of fixation method is multifaceted considering factors surrounding the fracture, the patient and the surgeon [42, 60, 61, 91]. Despite both methods being viable options in many cases, preference is typically given to the intramedullary device for several reasons: (1) its relative ease of use, (2) the reduced soft-tissue damage with its use compared to compression plating and (3) because the load-sharing properties of an intramedullary nail allow for immediate weight-bearing following surgery [42]. As such, seldom does the choice of fixation depend on whether or not the patient is a smoker.
Recently, a subset of CD34+ cells expressing Osteocalcin (OCN) has been described which migrate through the circulation and can differentiate into osteoblasts [24, 71]. These circulating osteoprogenitor cells (COPs) have been shown to mobilize in response to fractures [57] and it has been proposed that through modifying COPs, there may be novel therapeutic approaches for bony disorders such as osteoporosis and fracture healing [15]. Since nicotine is a potent vasoconstrictor [3] and CD34+ cells are known to be enriched for endothelial progenitor cells [65], we wanted to explore whether recruitment of this cell population would be inhibited by exposure to cigarette smoke.

**Primary Aims and Objectives**

In this work, we developed several primary aims:

(1) Establish a reproducible smoke-exposure protocol for use in the Sprague-Dawley rat and use this to test the primary and secondary healing pathways through use of an established bilateral femur-fracture rodent model.

(2) Determine whether smoking exerts a differential effect the two fracture healing pathways (endochondral and intramembranous ossification) through assessment of the fracture cartilage area.

(3) Measure the production of circulating osteoprogenitor cells and compare their induction under the condition of smoke exposure.

In order to accomplish these aims, we chose to use the Sprague-Dawley rat given our laboratory's experience using this species. Briefly, these animals offer large enough femora for generation of a reproducible fracture pattern which allows the use of an
intramedullary rod as well as compression plating. The use of these two fixation
techniques offer a unique approach and opportunity to study both intramembranous and
endochondral ossification within the same animal. Use of smaller rodent species prohibits
these surgical techniques given size limitations for adequate fixation via compression
plating. This species also demonstrates a similar fracture healing capacity as is seen in
humans, providing a robust and clinically relevant model for the study of fracture
healing.
CHAPTER TWO
RESEARCH DESIGN AND METHODS

Animal Experimentation and Surgical Model

Prior to initiation of this study, we received approval from the Medical University of South Carolina Institutional Animal Care and Use Committee. All procedures performed were in accordance with the Animal Scientific Procedures Act of 1986. The animal work described herein has been written to align with the ARRIVE guidelines [52]. In total, 46 male 6-month old (180 ± 23 days) pathogen-free Sprague-Dawley rats (average weight on arrival: 419 ± 37.9g) were acquired for use in a single experimental unit (Envigo, Indianapolis, IN, USA). An age of 6-months was chosen to allow for full maturation to skeletal maturity by the time of bilateral femur fracture surgery [43]. Males alone were used for this experiment, as estrogen-mediated hormonal fluctuations are known to impact bone-biology [28, 67, 92], potentially confounding our results. On arrival, these animals were randomly assigned as controls (n=23) or to receive smoke exposure (n=23). Two animals died during the course of this experiment: one as a result of an anesthetic overdose and another due to unsalvageable intraoperative fracture comminution and resulting failure of fixation. These animals were replaced by acquiring two additional animals (Envigo, Indianapolis, IN, USA) to ensure adequate statistical power for our analyses. Animals were randomly assigned to one of the following predetermined postoperative sacrifice timepoints: 10-days (n = 4/group), 28-days (n = 7/group), 3-months (n = 6/group) and 6-months (n = 6/group; Figure 2.1). The sample
numbers were determined in each group based on previous experiments using the same surgical model and experimental techniques. These numbers were determined using a power analysis (Sigma Stat. v3.5) with $\alpha = 0.05$ and a desired power of at least 80% with a moderate effect size (0.30) determined based on preliminary data generated with this same animal model.

Animals were housed in a specialized helicobacter-free institutional facility maintained by the Division of Laboratory Animal Resources. Briefly, animals were singly housed in ventilated racks with a reverse-osmosis auto watering system. Bedding material consisted of corn-cob material (Teklad 1/8th inch, Envigo, Indianapolis, IN) with the exception of time immediately following bilateral femur fracture surgery where the
animals were placed in a cellulose-based bedding (ALPHA-Dri™, Sinclair Coal & Oil, Florence, SC) for two weeks. Animals received standard enrichment with access to nesting materials throughout (Diamond Nest Teklad 6014C.CS). Animals received twelve-hour light/dark cycles and were maintained at a consistent ambient temperature of 22 °C. Throughout the course of the experiment, animals were allowed to eat standard rodent chow (Teklad 2918, Envigo, Indianapolis, IN) without restriction.

Smoke Exposure

Critical to the design of any well-controlled animal study is reproducibility and the act of generating reproducible smoke-exposures presents unique scientific challenges. In human smokers, the quality, quantity and composition of cigarette smoke may vary dramatically between individual smokers and even individual cigarettes [48]. This can be due to differences in tobacco composition (geographic location tobacco is grown, strain of plant, post-harvest processing), cigarette paper differences, cigarette size and presence of a filter (differences in fibrous density, tar adsorptive capacity) [27, 79]. Furthermore, in humans, the amount of smoke particulate that is inhaled is directly related to the following:

1. The duration and number of inhalations of mainstream smoke (defined as smoke particulate that is drawn through the filter of the cigarette) [55].

2. The concentration of ambient second-hand and side-stream (defined as smoke particulate that emanates directly from the burning end of the cigarette and is not drawn through the filter) smoke and the respective cumulative duration of exposure.
(3) The cumulative number of cigarettes smoked and total amount of ambient smoke particulate that a human is exposed to over a lifetime.

As such, we sought to develop a smoke-exposure protocol that could control for all of the aforementioned factors and allow for reproducible exposures throughout our experiments. To tackle the irregularities in composition of cigarettes both between brands of cigarettes as well as between batches, we identified the University of Kentucky’s Center for Tobacco Reference Products as a reliable producer of research-grade cigarettes. Since 1968, this center has produced cigarettes with a tobacco blends representative of modern-day (low tar) cigarette compositions [72]. Manufacturing occurs in bulk in order to ensure an even and consistent mixture of the three predominant tobacco sources. Analysis of their currently available reference cigarettes (3R4F) has shown that the quality and chemical contents of these cigarettes is no different from their prior batch (2R4F) [89]. Cigarettes were stored at 4ºC for preservation. At least 48h prior to use, cigarettes were placed in a humidifying chamber to reconstitute the tobacco. The plastic packaging was removed and the pack was opened to allow the humidity to access the cigarette ends. The humidifying chamber contained a solution of glycerin and water in a 0.76:0.24 ratio in order to establish a relative humidity of 60%.

We achieved reproducible lighting and puffing of cigarettes through use of a custom-built smoking machine (Teague Enterprises, Woodland, CA). This machine is able to automatically load, light, puff and eject cigarettes using a Bluetooth programmable software. A 10-slot carousel holds the cigarettes inside an airtight enclosure while the machine cycles the cigarettes between side-stream and mainstream
smoking positions. Air is drawn through the cigarettes, generating mainstream smoke through use of a one-way piston pump and is directed to an exhaust hose. Side-stream smoke is collected within the enclosure and is drawn through the exhaust hosing by means of a vacuum generated by an air blower. It is important to use this model, as side-stream smoke has been shown to be more toxic than mainstream smoke [83]. Mainstream and side-stream smoke is mixed thoroughly after it enters a cylindrical vortex. Smoke is then drawn through a combination from one to four chambers which can house up to six individual animals each. The rate at which smoke is drawn through these chambers is controlled through manipulation of a butterfly valve and adjusting the vacuum pressure applied to the smoke exposure system. After passing through the animal chambers, smoke is drawn through the blower and passes out of the machine.

In order to ensure that individual smoke exposures are consistent, the smoke concentration within each exposure chamber is measured on every run. Smoke is composed of both gas and solid-phase (particulate) substances. The particulate concentration can therefore be used as a measurement of smoke exposure in this system. Measurement of particulate concentration can be performed by drawing smoke through a pre-weighed 25mm Pallflex® membrane filter (EMFAB TX40H120-WW, Pall laboratories, Westborough, MA). Particulate matter is deposited on the filter paper as the air is drawn through it. The volume of air that is drawn through the filter paper is measured with an air flow meter and the concentration of smoke particulate can be calculated as follows:

\[
\text{Total Particulate Matter (TPM)} = \frac{\Delta \text{Filter Paper Mass (g)}}{\text{Volume of Air Filtered (m}^3\text{)}}
\]
A TPM target of 200mg/m$^3$ was used for individual exposures, as this smoke concentration has been shown to result in serum cotinine levels (a metabolic byproduct of nicotine; discussed later) analogous to those seen in heavy smokers [9]. For readings above or below 200 mg/m$^3$, airflow adjustments were made to approach our goal exposure: increased ambient airflow results in greater smoke dilution and a lower TPM reading, whereas decreased ambient airflow results in increased TPM. Measurements were taken 10-minutes after turning on the smoking machine in order for the smoke levels to equilibrate.

To simulate the frequency of smoke exposures seen in human patients, we designed each smoke exposure to occur for one-hour in duration with two exposures on each weekday and one exposure on each weekend day (n = 12/week). Between exposures, smoke was released from the smoking chambers and animals were allowed to breathe room air for one hour before beginning a second smoking session. During each run, a total of 30 3R4F cigarettes were loaded in the magazine and the machine was programmed to sequentially light and puff five cigarettes per cycle for a total of six cycles. During each cycle air was drawn through the cigarettes by the puff pump for two second durations to mimic the average puff duration of a human smoker. Each cigarette received ten puff cycles prior to being ejected from the machine. This number of cycles resulted in an even burn of the tobacco but not the cigarette filter. The machine was programmed to automatically shut down after a 60-minute smoking session.

**Enzyme-Linked Immunosorbent Assays (ELISA)**
In humans, cigarette smoke exposure is not measured in TPM, rather it is difficult to quantify. Typically, a history of smoke exposure is gained through patient-reported smoking habits which allows calculation of a patient’s pack-year history, which is defined as the number of packs of cigarettes smoked per day per years (e.g. a patient that has smoked a half-pack of cigarettes per day for six years would have a three pack-year history). However, self-reported smoking habits rarely reflect a patient’s true exposure to cigarette smoke [68]. Clinically, the degree to which a patient is exposed to cigarette smoke can be quantified through measurement of their serum cotinine levels, a metabolic breakdown product of nicotine [4, 44, 53, 68]. The purpose for correlating cigarette smoke exposure with serum cotinine levels rather than nicotine levels is due to the increased half-life and thus, reliability in cotinine measuring compared to nicotine (16h vs 0.9-1.1h) [4, 44, 59, 64, 76, 95]. We aimed to achieve serum cotinine levels of 300ng/mL, as this has been shown to be the average cotinine level in humans that smoke one pack (20 cigarettes) per day [5]. Prior research in a rat smoke-exposure model has shown that a TPM exposure of 200mg/m³ results in serum cotinine levels of 332.8 ± 20.86 ng/mL [9]; as such, we aimed to achieve 200mg/m³ TPM readings on each of our exposures. To verify that this concentration of cigarette smoke would replicate the cotinine levels seen in humans, we tested the rodent serum cotinine levels after 5 days of active smoke exposure. Approximately 20 hours following their most recent smoke exposure, rodents were induced via 4% isoflurane 2L/min O₂ and maintained on a nosecone with 2% isoflurane 1L/min O₂. Tails were wiped clean with 70% v/v isopropyl alcohol and warmed with a 45°C isothermal heating pad for 1 minute. Lateral tail veins
were identified and 100µl of blood was drawn using a 18g needle. Samples were centrifuged at 14,000rpm at 4°C and the plasma supernatant collected for cotinine measurement. A cotinine-specific Enzyme-Linked Immunosorbent Assay (ELISA) was performed according to the manufacturer’s instructions (MBS580061, Mybiosource). Briefly, rat plasma was diluted 5x in 1x phosphate buffered saline (PBS), samples were run in triplicate to ensure accuracy and absorbance was measured at 450nm (ELX808, BioTek). Cotinine measurements were calculated and expressed as means with standard deviations. In order to calculate serum cotinine levels during the time of the exposure, the following half-life formula was utilized:

\[
N(t) = N_0 \left(\frac{1}{2}\right)^{t/t_{1/2}}
\]

\[N(t) = Serum\ cotinine\ level\ at\ time\ t\ (hours);\ t_{1/2} = 16h, cotinine\ half\ life\]

Adequate nutrition is key for fracture healing to occur [31]. Because cigarette smoke is known to cause weight loss [11] we monitored rodent total body weight daily on initiation of smoke exposure and continued this for 56 days following fracture surgery. To further ensure that animals were not malnourished rodent transthyretin plasma levels were assessed via ELISA either immediately preoperatively or on postoperative day 10. Transthyretin, also known as prealbumin, is a well-characterized nutritional marker with a short half-life (29h) [21, 45, 46]; as such, postoperative day 10 was chosen as a comparison since this length of time would allow the transthyretin levels adequate time to adjust to the bilateral femur fracture injury [21]. The transthyretin ELISA was performed
according to the manufacturer’s instructions (OKIA00159, Aviva Systems Biology, San Diego, CA) in duplicate to ensure internal consistency and comparisons made via one-way ANOVA.
Femoral Implant Design

In order to provide both rigid fixation and fracture compression to allow intramembranous ossification to proceed, we developed a technique in the Sprague-Dawley rat using a plate and screws (unpublished data). We obtained 1.5x100mm straight hole plates (246.19, Synthes, Monument, CO) and cut them into five-hole, 25mm segments. This five-hole design allowed us to create a fracture about the center hole of the plate while providing two-screw fixation both proximally and distally to the fracture site. Eccentric drilling, away from the fracture site, in each position allowed us to achieve fracture compression while tightening 1.5x6mm cortical screws (200.806, Synthes, Synthes, Monument, CO).

Prior studies using intramedullary nails in the rat have used simple wire fixation, which fails to control for rotational instability. As such, we sought to design an implant that would allow for sufficient movement to induce endochondral ossification, but would not be subject to excessive rotatory instability. To do this, we obtained custom-made 1.6mm OD x 0.28mm wall x 24mm long 304-stainless steel hypodermic needles (B&B Precision Wire EDM, INC, Forest Hill, MD; Figure 2.2).

Figure 2.2- Intramedullary Nail and Guide Design: Photograph of intramedullary device with drill guide in place. The 0.76mm drill bit (right) demonstrates the alignment between the drill guide and the distal interlocking hole.
Two parallel 1.1mm interlocking holes were made through the needle, 20mm apart. A 90º bend in the needle introduced an elbow that allows for use of a custom drill guide during nail placement. After both the proximal and distal interlocking screws are affixed in the femur, the drill guide can be easily removed and the nail can be cut at the 90º bend.

Bilateral Femur Fracture Surgical Procedure

Following induction and maintenance of isoflurane anesthesia using, animals were given a single subcutaneous dose of 1.0mg/kg buprenorphine-SR LAB (Zoopharm, Windsor, CO) preoperatively for 72 hours of analgesia as well as a single 10mg/kg dose of enrofloxacin (Zoopharm, Windsor, CO) for infection prophylaxis. Enrofloxacin was chosen given its long half-life and ease of administration. Following all surgeries, animals were monitored twice daily for surgical complications. Animals were monitored daily for signs of pain for the first week following surgery and daily thereafter, until the time of euthanasia. If animals demonstrated excessive pain postoperatively, additional analgesics were considered (buprenorphine), and veterinarian consultation was obtained. Objective signs of animal distress included porphyrin staining about the eyes, decreased activity, poor ambulation, decreased urine/feces output, aggression on handling or self-mutilation. Incisions were monitored for signs of inflammation, infection and wound dehiscence until fully healed by postoperative week four. Animal bodyweight was monitored for excessive weight loss following surgery with greater than 20% weight loss as a cutoff for unscheduled euthanasia.
Animals were prepared for surgery by removing hair ventrally and dorsally from the distal hind limbs to the thorax and sanitizing the skin with an alternating scrub of betadine and 70% v/v isopropyl alcohol. A 4 cm longitudinal incision was made through the skin on the lateral aspect of the thigh to expose the underlying muscle and fascia. The lateral femur was accessed through sharp dissection through the anterolateral fascial planes and the surface of the bone was carefully exposed. For the compression plate technique, a power drill was used to eccentrically drill 1.1mm bicortical holes through the first and last holes of a 1.5-mm thick, 5-hole stainless steel plate positioned on the anterior aspect of the femur. Two 1.5mm x 6mm cortical screws were used to provisionally hold the plate in position while making several bicortical passes through the center of the femur with a 0.76x14 mm bit, using the center hole of the plate for reference. The plate was loosened, and a transverse fracture was made through the pre-drilled holes using a 6.35-mm osteotome and mallet. The distal screws were tightened, while both reducing and compressing the fracture fragments. Additional compression was achieved by placing eccentric screws in the remaining two holes immediately adjacent to the fracture. Prior to closure, the fracture reduction was inspected for stability, gaps and comminution, with any intraoperative complications noted. Fascial closure was achieved through use of a running 4-0 Vicryl™ (Ethicon) suture and skin was closed through use of staples (Kent Scientific).

For intramedullary nail fixation, a transverse fracture was created using the same technique as above. The proximal femoral medullary canal was then reamed via retrograde hand-drilling using serially increasing size drill bits (1.3 mm, 1.5 mm, and 1.8
mm), exiting through the proximal femur about the greater trochanter. A 0.8-mm K-wire was used as a guide wire to allow a custom-made intramedullary nail, previously developed by our laboratory (described elsewhere), to be inserted antegrade through the greater trochanter and into the medullary canal. A custom-made drill guide was then used to drill 0.76 mm holes through the interlocking holes in the nail. An interlocking screw was placed in the distal position, the fracture fragments were manually compressed, and the proximal locking screw was placed secure the fracture. Prior studies have failed to use an interlocking technique for nail fracture fixation [8, 23, 69]. Rotational instability causes aberrant ambulation of quadrupeds and thus providing interlocking fixation is critically important for a clinically-relevant model. Fascia and skin were closed similarly to above. Serial X-rays were performed (Faxitron, LX-60, Tucson, AZ) immediately postoperatively to assess fracture fixation as well as on post-operative days 4, 10, 21, 28 and in two-week increments thereafter to monitor the progression of osseous healing.

MicroCT Assessment of Callus Volume

In order to determine if smoking had an effect on one or both of the distinct pathways of fracture healing, microCT was performed on femora harvested at 28 days, 3 months and 6 months postoperatively. MicroCT was used to measure the total volume of the calcified callus, in order to represent the endochondral ossification pathway. Images were taken with a Scanco μCT40 system (Scanco Medical, Wayne, PA, USA) using a 10 μm isotropic voxel size (70 kVp, 114 mA). A three-dimensional (3-D) volumetric reconstruction technique was used to measure the calcified callus volume (CV), the
femoral bone volume (BV), and the calcified callus volume ratio (CV/BV). The CV/BV ratio was used for all analyses in order to control for anatomic differences in femoral size between animals. Three different contours were manually drawn on a series of microCT images which included: (1) The calcified callus external to the bone, (2) the callus present within the femoral canal, and (3) the femur alone. The overall volume of calcified callus was calculated by adding both the callus external to the bone and the callus within the medullary canal using the Scanco µCT software.

Quantitative Histomorphometry

A further assessment of endochondral ossification was performed through histomorphometric measurement of the cartilaginous callus. Femurs were fixed in 10% formalin, decalcified in 18% formic acid, and then embedded in paraffin. Tissue sections that maximize the fracture cross sections were obtained, mounted on slides, and stained with either safranin-O and fast green or hematoxylin and eosin. Safranin-O/fast green staining was chosen given its ability to stain proteoglycans and highlight cartilage [90], with a large distinction between cartilage (red) and non-cartilage tissues (green). Serial 2x histologic photographs were taken using an Olympus BX-40 microscope and stitched together using Fiji (NIH) software [84]. Quantification of cartilage area at the fracture site was performed using ImageJ2 (NIH) software [80]. Briefly, cartilage was identified by selecting pixels with red-hue and measured and then pixel numbers were converted into area (mm²). All samples were quantified by the same author (RAR) to ensure consistency.
Statistical Analysis

For early osseous healing, the cartilaginous area and femoral callus volume were examined to detect differences attributable to the fixation method (plate vs nail) and exposure (smoke vs control). We conducted two separate statistical analyses: (1) comparison of the cartilage area histomorphometry, (2) comparison of the calcified callus volume ratios (CV/BV) from microCT which reflect changes in the size of the calcified callus. We used IBM SPSS Statistics-Version 24 (Armonk, NY, USA) to perform all statistical comparisons. A two-way analysis of variance (ANOVA) was used to test the interaction between the fixation method and between the exposure group (smoke vs control). Fisher’s least significant difference (LSD) was performed post-hoc to compare individual interactions within the groups. Significance was defined as $\alpha < 0.050$. 
CHAPTER THREE

THE LOCAL EFFECTS OF CIGARETTE SMOKE ON FRACTURE HEALING

Cigarette Smoke Exposure

Rodents were subjected to smoke exposure daily with Total Particulate Matter (TPM) calculated for every run. The average TPM was $200.6 \pm 73.0 \text{ mg/m}^3$ (95% CI: 193.9 - 207.3, Range: 61 - 704). Exposures were limited to a maximum of 12 animals simultaneously, given the size restrictions of our smoke-exposure enclosure. As such, we performed a one-way ANOVA to assess whether animals received higher or lower smoke exposures on average from one another. There were no statistical outliers with regard to average smoke exposure (one-way ANOVA: $p = 0.061$, Figure 3.1).

Figure 3.1: Average TPM Exposures by Animal and Sacrifice Timepoint

Animal Harvest Timepoints:

- 10 Days
- 28 Days
- 3 Months
- 6 Months

Error Bars: 95% CI
In order to further ensure that our smoke exposures were clinically-relevant, we performed an ELISA on rodent serum cotinine following 5 days of smoke exposure, 20 hours after their most recent smoke exposure. The serum cotinine levels were 155 ± 34ng/ml at 20 hours and, based on the half-life of cotinine, was estimated at 368 ± 82ng/mL immediately following smoke exposure.

**Surgical Outcomes**

Of the 46 animals that underwent bilateral femur fracture surgery, 44 animals survived and two of the control animals did not (Figure 2.1). One animal died intraoperatively as a result of an unexpected anesthesia overdose. The other animal required early euthanasia due to the highly comminuted fracture that was made intraoperatively. The comminution was so severe that it prevented adequate nail fixation despite attempts at using additional adjunct fixation (cerclage sutures). As such, the animal was euthanized prior to completing the surgical procedure. In order to maintain adequate statistical power, two additional animals were purchased to replace these lost animals, and as such these animals could not be randomized. In the 46 animals that survived surgery, all were able to adequately ambulate immediately following surgery without apparent gait impairment. X-rays were obtained postoperatively which demonstrated adequate fracture reduction both in the plated and nailed femurs and over time displayed progression of fracture healing as evidenced by bony callus formation and diminished fracture radiolucency (Figure 3.2). On removal of skin staples at postoperative day 10, no animals exhibited wound dehiscence.
**Figure 3.2:** Serial postoperative radiographs of rodent femora taken at day 10 and at 3 months to monitor for osseous healing and hardware failure are shown. Both anterior-posterior (AP) and lateral (Lat.) X-rays are displayed of both plated and nailed specimen. The left column demonstrates representative images from a control animal where the right column demonstrates a smoke-exposed animal.
Effects of Cigarette Smoke on Nutritional Status

Throughout the course of this experiment, rodents were assessed for weight loss. Bodyweights were recorded daily and plotted relative to the animal’s bodyweight 28 days prior to surgery (Figure 3.3). Prior to smoke-exposures, control and interventional animals had bodyweights of 442 ± 10.0g and 431 ± 10.0g, respectively (p = 0.447, Table 3.1). Preoperatively, smoke-exposed animals exhibited weight loss after 10 days of smoke-exposure, whereas control animals continued to gain bodyweight (Mean
difference: 50.1g, 95% CI: 19.4, 80.8, p = 0.002) and this trend throughout the duration of smoke exposure. By one month of cessation of smoke exposure (day 84, Table 3.1), there was no difference in bodyweight between smoke-exposed animals and controls (Mean difference: -1.42, 95% CI -29.5, 26.6; p = 0.921). Compared to their bodyweight on day zero, smoke-exposed animals exhibited weight loss one month following surgery (day 42, mean difference: -62.4g, 95% CI: -88.7-36.2g, p < 0.001; Table 3.2), which persisted for one month following smoking-cessation (day 56, mean difference: -45.1g, 95% CI: -71.3-18.9g, p < 0.001).

Table 3.1: Rodent Bodyweight in Response to Smoke Exposure

<table>
<thead>
<tr>
<th>Relative Smoking Day</th>
<th>Control Mean ± SD</th>
<th>Smoke-Exposed Mean ± SD</th>
<th>Mean Difference (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>442 ± 10.0</td>
<td>431 ± 10.0</td>
<td>10.8 (-17.2, 38.9)</td>
<td>0.447</td>
</tr>
<tr>
<td>10</td>
<td>456 ± 11.0</td>
<td>406 ± 11.0</td>
<td>50.1 (19.4, 80.8)</td>
<td>0.002 **</td>
</tr>
<tr>
<td>28</td>
<td>450 ± 7.25</td>
<td>420 ± 7.25</td>
<td>31.0 (10.7, 51.2)</td>
<td>0.003 **</td>
</tr>
<tr>
<td>42</td>
<td>427 ± 20.1</td>
<td>370 ± 8.70</td>
<td>57.5 (14.3, 100.7)</td>
<td>0.009 **</td>
</tr>
<tr>
<td>56</td>
<td>437 ± 8.44</td>
<td>387 ± 8.70</td>
<td>50.2 (26.3, 74.2)</td>
<td>&lt;0.001 ***</td>
</tr>
<tr>
<td>84</td>
<td>448 ± 10.0</td>
<td>449 ± 10.0</td>
<td>-1.42 (-29.5, 26.6)</td>
<td>0.921</td>
</tr>
</tbody>
</table>

Two-way ANOVA:

<table>
<thead>
<tr>
<th></th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative Smoking Day vs Exposure (Control vs Smoke)</td>
<td>0.030 *</td>
</tr>
<tr>
<td>Relative Smoking Day</td>
<td>&lt;0.001 ***</td>
</tr>
<tr>
<td>Exposure (Control vs Smoke)</td>
<td>&lt;0.001 ***</td>
</tr>
</tbody>
</table>

Table 3.1: Animal bodyweights (grams) were obtained and compared between control and smoke-exposed animals. Comparisons were made relative to the starting date of smoke exposure (day zero). Day 28 corresponds to the date of surgery. Day 42 signifies the weight loss nadir and day 56 signifies the last day of smoke exposure. *Signifies p < 0.050, ** signifies p < 0.010 and *** signifies p < 0.001.
Given the decreased bodyweight seen in the smoke-exposed animals, we sought to determine whether this was due to an anorexogenic effect of the smoke exposure. As such, we measured the amount of food consumption by measuring the animals average dry food consumption. We did this though calculating the differences in food weights every forth day (Figure 3.4). Compared to control animals, smoke-exposed animals consumed less food following the initiation of smoke exposure (day 10, mean difference: -6.56, 95% CI: -9.54, -3.59; p < 0.001, Table 3.3). However, there was no difference in food consumption following surgery. Notably, following the cessation of smoke

Table 3.2: Animal bodyweights (grams) were obtained from both control and smoke-exposed animals and compared over time to their pre-smoking level (day zero). Day 28 corresponds to the date of surgery. Day 42 signifies the weight loss nadir and day 56 signifies the last day of smoke exposure. *Signifies p < 0.050, ** signifies p < 0.010 and *** signifies p < 0.001.

<table>
<thead>
<tr>
<th>Table 3.2: Rodent Bodyweight Changes Over Time</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control Animals</strong></td>
</tr>
<tr>
<td><strong>Comparisons to Day Zero</strong></td>
</tr>
<tr>
<td>Relative Smoking Day</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>28</td>
</tr>
<tr>
<td>42</td>
</tr>
<tr>
<td>56</td>
</tr>
<tr>
<td>84</td>
</tr>
</tbody>
</table>

| **Smoke-Exposed Animals**                     |
| **Comparisons to Day Zero**                   |
| Relative Smoking Day | Mean Difference (95% CI) | p-value |
| 10       | -26.0 (-55.4, 3.40)      | 0.083   |
| 28       | -12.2 (-36.7, 12.3)      | 0.327   |
| 42       | -62.4 (-88.7, 36.2)      | <0.001  *** |
| 56       | -45.1 (-71.3, 18.9)      | <0.001  *** |
| 84       | 17.9 (-10.1, 46.0)       | 0.209   |
exposure, smoke-exposed animals consumed more food relative to control animals, however this did not reach statistical significance (day 84, mean difference: 4.75, 95% CI: -0.41, 9.91; p = 0.071).

Since it was unclear whether or not this reduced food consumption would result in malnutrition, we sought to measure the animals’ serum transthyretin, a marker nutritional status. To do this, blood samples were taken either after 5 days of smoke exposure or on postoperative day 10 and transthyretin levels were assessed via ELISA. Neither surgery,
Figure 2.5: Plasma Transthyretin Levels by Exposure and Operative Status

Figure 3.5: Transthyretin ELISA assays were performed on animals both 23-days preoperatively and 10-days postoperatively to assess their overall catabolic states. No differences were observed between smoke-exposed and control animals. Bolded horizontal lines represent mean transthyretin levels and bottom and top ends of the boxes extend to the 25th and 75th quartiles, respectively. Whiskers represent the extreme values.
Effects of Cigarette Smoke Exposure on Endochondral Ossification:

The Cartilaginous Callus

In order to measure the effects of cigarette smoke on the endochondral ossification bone healing pathway, we first assessed the production of the initial cartilaginous intermediary at 10-days postoperatively. To do this, we performed femoral tissue staining using hematoxylin and eosin to fully characterize the fracture site histologic morphology followed by performing safranin-O staining to stain for cartilaginous proteoglycans (Figure 3.6). Quantification was performed using ImageJ and we found that for control animals, cartilage area in the nailed specimen was greater than that of the plate (Figure 3.7), consistent with induction of endochondral ossification.

Figure 2.6- 10-Day Cartilage Formation via Safranin-O Staining: Safranin-O staining was performed on animals sacrificed on postoperative day 10. Histomorphometry was performed to quantify the absolute cartilage area (red staining) per slide. Representative tissue sections are shown here, both with plate and nailed specimen.
Table 4. However, animals exposed to cigarette smoke exhibited a marked reduction in cartilage area around nailed femoral specimen [Mean difference (control nail vs smoke-exposed nail): 0.272mm², 95% CI (0.096, 0.0447), p = 0.007]. As of the time
of this writing, the histologic assessment of later time points (28 days, 3 and 6 months) have yet to occur.

Table 3.4: Cartilage Histomorphometry by Fixation Method and Smoke Exposure

<table>
<thead>
<tr>
<th>Cartilage Area (mm²)</th>
<th>Plate (n = 6)</th>
<th>Nail (n = 7)</th>
<th>Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean Difference (95% CI)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 Days</td>
<td>0.158 ± 0.109</td>
<td>0.380 ± 0.132</td>
<td>0.222 (0.023, 0.420)</td>
</tr>
<tr>
<td>Smoke-Exposed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 Days</td>
<td>0.145 ± 0.069</td>
<td>0.108 ± 0.830</td>
<td>0.037 (-0.138, 0.212)</td>
</tr>
</tbody>
</table>

2-way ANOVA:

<table>
<thead>
<tr>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixation (Plate vs Nail) vs Exposure (Smoke vs Control)</td>
</tr>
</tbody>
</table>

Table 3.4: Quantification of cartilaginous callus via Safranin-O staining around the fracture site. Cartilage area (mm²) is expressed as means with standard deviations. Comparisons were made through a two-way ANOVA, considering both the effects of fixation method as well as exposure (smoke vs control). Post-hoc comparisons were made using Fisher’s LSD.

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CHAPTER FOUR

THE SYSTEMIC EFFECTS OF CIGARETTE SMOKE ON FRACTURE HEALING

Effects of Cigarette Smoke on Basal Circulating Osteoprogenitor Cells

We first sought to determine whether cigarette smoke-exposure alone had any effect on the basal production of circulating osteoprogenitor cells prior to fracture surgery. To do this, we obtained plasma samples were collected from rats exposed to either one, two or four weeks of daily smoke exposure and assessed for circulating osteoprogenitor cells (COPs) via flow cytometry (Figure 4.1). All preoperative blood was obtained and processed via flow cytometry on the same day in order to minimize inter-batch variation. The flow cytometry gating strategy is displayed in Figure 4.2. Briefly, circulating osteoprogenitor cells were identified as cells that stained positively for both CD34, a hematopoietic cell marker, and osteocalcin (OCN), a secretory protein of osteoblasts, similar to prior studies [73, 74]. We found that neither one, two or four

---

**Figure 4.1:** This flowchart describes the blood-draw timepoints for circulating osteoprogenitor (COP) analysis prior to bilateral femur fracture surgery. Vertical lines indicate the relative day that animals underwent tail-vein blood draws for flow-cytometry. *Operative timeline is expressed as days relative to the first day of smoke exposure, which is defined as day 0.*
Figure 4.2 - Gaiting Strategy to Identify Circulating Osteoprogenitor Cells: First, lymphocytes were selected based on forward-scatter (FSC-A) and side-scatter (SSC-A) pattern. Live cells were identified through low propidium iodide (PI) update. Cell enumeration was performed using 123count eBeads. Circulating osteoprogenitor cells were identified as both CD34 and Osteocalcin (OCN) double positive cells. FMO - Fluorescence Minus One, FITC - Fluorescein Isothiocyanate, APC - Allophycocyanin.
weeks of smoke-exposure had any effect on either the basal number or percentage of osteoprogenitors in rat peripheral blood (Figure 4.3).
Figure 4.3- Effects of Cigarette Smoke on Basal Circulating Osteoprogenitor Cells: Animals (n = 4/group) were exposed to cigarette smoke for either 1, 2 or 4 weeks prior to blood draw. No significant differences were observed in the basal production of osteoprogenitor cells (CD34+, OCN+). Cells are expressed as both absolute number (left) and percentage (right).
Effects of Cigarette Smoke on the Mobilization of Circulating Osteoprogenitor Cells Following Fracture

In order to assess whether cigarette smoke exposure affects the mobilization of COPs following fracture, we performed the following experiment. Animals were subjected to either daily smoke exposure or were designated as control animals as previously described (Figure 2.1), however, a subset of these animals (n = 12/group) underwent serial tail-vein blood draws to assess for COPs following bilateral femur fracture surgery (Figure 4.4). Blood draws were performed on postoperative days: 4, 14, 21, 28 and 35, and flow cytometry was performed using the same gating strategy as described above. Not all animals underwent blood draw at postoperative day 35 as their pre-determined sacrifice was set to 28 days to achieve our primary study objectives.

Similar to our results in Figure 4.3, prior to fracture surgery cigarette smoke had no effect on COPs (Figure 4.5, Appendix 1.1). However, following fracture surgery, smoke exposure decreased the percentage of COPs at 4 days postoperatively (Early Post-Fx timepoint, p = 0.0470, Appendix 1.2). At two-weeks following surgery, smoke-
Figure 4.5- Temporal effects of cigarette smoke on circulating osteoprogenitor cells following bilateral femur fracture surgery. Serial blood draws were performed on animals following bilateral femur fracture surgery. Cells are expressed as both absolute number (left) and relative percentages (right). Smoke-exposed animals demonstrated less osteoprogenitor cells from postoperative day two through two weeks postoperatively but demonstrated a greater number of osteoprogenitor cells at week four. Smoke exposure ceased at four weeks postoperatively. This trend was the same for percentage of osteoprogenitor cells with the exception of the early post-fracture timepoint. *Denotes significant difference between smoke-exposed and control animals. Fx – Fracture, OCN- Osteocalcin, SEM- Standard error of the mean.
exposure resulted in both a decrease in the absolute number of COPs as well as the percentage of COPs compared to control animals (Number: p = 0.0114, Percentage: p = 0.0143, Appendix 1.3). There was no difference in COP levels at 3 weeks following surgery (Appendix 1.4), but at four weeks the smoke-exposed animals demonstrated both a greater number and percentage of COPs compared to the controls (Number: p = 0.0412, Percentage: p = 0.0053, Appendix 1.5). At five weeks following fracture surgery, there was no difference in COPs in smoke-exposed animals vs controls.
CHAPTER FIVE

DISCUSSION

This work represents the successful implementation of a cigarette smoke-exposure protocol in a bilateral femur fracture model. Few investigators have developed intramembranous ossification models, given the difficulty in producing stable anatomic fixation with interfragmentary compression [39, 88]. As opposed to prior studies, the bilateral fracture technique allows us to uniquely compare the effects of cigarette smoke on the two healing pathways in the same animal. Variation between animals can be substantial, and so using both techniques in the same animal, we are able to minimize intra-animal variation and we are also able to substantially reduce the number of animals required for such comparisons. Furthermore, compared to other stabilized fracture techniques [97], the use of static interlocking screws within the intramedullary nail provided adequate micromotion, which is required for induction of endochondral ossification [14], while providing adequate rotatory stabilization, which permitted normal rodent ambulation immediately postoperatively. As such, all animals successfully exhibited radiographic healing using this novel approach.

Fracture healing is complex and proceeds with the development by a fracture hematoma, infiltration of immune cells, cellular regeneration and finally tissue remodeling [49] and cigarette smoke-mediated inhibition of any one of these stages may result in impaired fracture healing. Prior studies have investigated the effects of nicotine alone on fracture healing given its ease of administration and known vasoactive properties [19, 58, 77]. Consistent with this study, Raikin, et al. showed that tibial
fractures treated with plates in rabbits had reduced radiographic callus formation in response to nicotine exposure and exhibited lower biomechanical strength at 8 weeks, suggesting impaired endochondral healing [77]; however, compression plate fixation should produce minimal callus due to their rigid fixation. Since these findings were not followed-up with histology it is unclear whether the differences in this study were due to an impact on endochondral or on intramembranous ossification. Several studies have explored nicotine’s affect on the cholinergic anti-inflammatory pathway and its role in reducing tumor-necrosis factor alpha (TNFα) [13]. In TNFα-deficient mice, both the recruitment of progenitor cells and apoptosis of cartilaginous cells are delayed resulting in impaired endochondral callus formation [30]. These studies provide a framework for understanding how nicotine may affect the inflammatory phase of fracture healing; however, nicotine-based studies are somewhat difficult to interpret, as there are many more biologically active compounds in cigarette smoke than nicotine alone [3, 58]. This may be particularly relevant for bone healing, as Skott, et al. showed that tobacco extract alone and in combination with nicotine impaired fracture healing as assessed by mechanical testing of the rat femur [86]. Importantly, an in vitro study by Guillihorn, et al., demonstrated that nicotine and cigarette smoke condensate had completely contradictory effects on osteoblast activity, with nicotine activating osteoblasts but smoke condensate inhibiting such function [34]. Furthermore, extremely high concentrations of nicotine, far greater than even the heaviest smokers, has been shown to have no effect on the cellular function or activity in chick tibias cultured in vitro [29, 38]. As such, we
sought to as closely replicate the true biologic effects of smoking cigarettes on osseous healing by developing an accurate in vivo smoke-exposure protocol.

The production of a reliable and reproducible smoke-exposure apparatus is challenging. Many different strategies have been taken with this goal in mind [9, 32, 77, 93]; however most studies opt to use simpler approaches through administering either nicotine alone or in combination with tobacco extract [77, 86]. In this study we were able to procure a custom-build smoking apparatus and develop a reproducible smoke-exposure protocol for the study of the effects of smoking on bone healing. Furthermore, we were able to validate this smoke exposure model to make it comparable to human patients that smoke one pack of cigarettes per day [5].

Cigarette smoke exposure is known to induce weight loss through anorexogenic effects mediated by reduced hypothalamic neuropeptide Y signaling [10, 51]. Similar to previous studies, smoke-exposed rodents lost a significant amount of weight but quickly caught up to control animals following smoking cessation [94]. Fracture nonunions have been associated with changes in serum nutritional parameters such as albumin [2, 78], and as such, we measured serum transthyretin levels to determine whether smoke-exposure exerted its inhibitory effect on fracture healing through causing malnutrition. However, these levels were no different between smoke-exposed and control animals. Transthyretin was chosen since it is a sensitive marker to both acute caloric and/or protein malnutrition. Since these levels were normal, it is unlikely that the animals experienced any significant malnutrition in response to smoke exposure. However, a
future study introducing a third food-restricted arm to further explore this possibility should be considered.

Using a custom-built cigarette smoke exposure apparatus and a twice-daily exposure approach, we found that cigarette smoke exposure inhibited endochondral ossification at 10 days, as seen by a reduction in cartilage formation around the smoke-exposed nailed specimen. Such a mechanism could give insight into why patients who smoke experience greater rates of nonunion and longer time to union than nonsmokers. The lack of a cartilaginous intermediate results in loss of provisional stabilization as well as loss of the necessary scaffolding for subsequent calcification and bony remodeling. This implies that treatment of fractures in actively smoking patients may be optimized through use of compression plating rather than intramedullary nailing since this would harness the intramembranous rather than endochondral healing pathway. It should be noted that treatment using an intramedullary nail often has several distinct advantages over compression plating, particularly for diaphyseal fractures [6]. Intramedullary devices are load-sharing and permit immediate weightbearing following treatment. This allows for patients to immediately begin rehabilitation following surgery, whereas compression plating requires immobilization until fracture union is achieved. Additionally, intramedullary nails often allow for a less-invasive treatment approach, particularly for femoral diaphyseal fractures. Compression plating often requires extensive tissue dissection with periosteal stripping in order to achieve an optimal fracture reduction, which causes increased patient morbidity at the surgical site.
COPs are a recently described cell population that have been implicated in fracture healing, osteoporosis and even the process of arterial atherosclerosis [15, 24, 25, 74], although their role in fracture healing and nonunions is still not well understood. Given that these cells are increased during pubertal growth periods as well as following fractures [25], there has been much interest in determining what role, if any, these cells play in the bone repair and regeneration processes. Since cigarette smoke induces inflammation and COPs are lymphocyte-derived, we wanted to first see whether smoke had an effect on the baseline production of COPs. Suprisingly, we found that daily exposure to cigarette smoke for up to one month had no significant effect on basal COP production. However, since COPs are known to mobilize in response to fractures, we hypothesized that the cigarette smoke may impair COP mobilization, resulting in fewer COPs arriving at the site of injury. We showed that cigarette smoke caused impaired early recruitment, whereas paradoxically later recruitment was increased despite continued smoke exposure. It is unclear how the cigarette smoke specifically impairs the induction of these cells, as smoke exposure had no effect on overall CD34 cell production, suggesting that overall inflammation is not impaired. However, the delayed timing of recruitment may be one of the mechanisms by which fracture healing is impaired in smokers, in that COPs arriving beyond a certain window may not play as an effective role in fracture healing.

This study has several limitations. In our comparison between the two fracture healing pathways, we focus on the effects of cigarette smoke on endochondral ossification; however, we have not directly measured the effect of cigarette smoke on
intramembranous ossification. Four-point mechanical testing allows us to compare the mechanical properties of femurs healed through each pathway (intramembranous vs endochondral) and it also gives us an opportunity to compare the strength gained through an individual pathway depending on exposure (smoke exposed vs control). As such, we can make inferences on the effect of cigarette smoke on intramembranous ossification; however this method lacks mechanistic details. Future work using this model could further define this impact through specific histologic staining of woven bone formed via the intramembranous pathway. Second, for the intramedullary nail surgery, we use an open-fracture approach. This is required in order for us to be able to provide adequate fixation with the interlocking screws. However, clinically fractures treated with intramedullary nails are typically achieved using a minimally-invasive technique. Thus, our model violates the fracture hematoma initially, likely washing away some of the inflammatory mediators and cells required during the initial healing response. Other investigators have developed closed fracture models with simple intramedullary pin fixation [7, 12, 63, 69]. These fractures exhibit rotatory instability and the surgical approach also often requires violation of the knee joint with resulting damage to the articular cartilage and significantly altered ambulation. Future work by our laboratory will aim to further optimize our surgical technique through providing surgical fixation using a minimally-invasive approach with percutaneous interlocking screws. Proximal to distal placement of the intramedullary device through the greater trochanter will avoid violating the knee joint thereby minimally impacting rodent ambulation. Finally, our assessment of circulating osteoprogenitor cells is only descriptive and cannot be used to
definitely demonstrate the mechanism of impaired fracture healing. Future work demonstrating histologically impaired recruitment of COPs at the fracture site would indeed confirm their late arrival. Furthermore, a study which reduces fracture nonunions in response to a pharmacologically-mediated increase in COPs (such as with AMD3100 or local use of granulocyte colony stimulating factor) [66] would provide a greater mechanistic foundation for understanding smoke-induced nonunions.
CHAPTER SIX

CONCLUSIONS AND FUTURE DIRECTIONS

This study has both important clinical and future research implications. First, clinically, these results suggest that for patients who are active smokers, orthopaedic surgeons should carefully weigh the pros and cons of the different fracture treatment approaches given the apparently selective inhibition of fracture healing via the endochondral ossification pathway. These results suggest that in the appropriately selected patient, rigid plate fixation may offer an optimal treatment approach for the patient at elevated risk for developing a fracture nonunion. As with any animal study, further validation must be performed both in this model and in humans prior to offering any definitive clinical recommendation. Furthermore, future studies using both male and female specimen ought to be used in order to further define the role of sex hormones in this fracture healing process. In order to further define the optimal fixation method under the condition of smoke exposure, future work from our laboratory will focus on the long-term implications of smoking on fracture healing. Future work will involve histomorphometry of the cartilage area, microCT of the callus volume and 4-point mechanical testing at 28 days, 3 months and 6 months postoperatively, as the survival of animals in these timepoints is still ongoing. These results will further define whether smoking causes a completely inhibitory effect on fracture healing or whether it simply decreases the rate at which endochondral healing can occur. We will also explore the histologic recruitment of COPs through use of immunofluorescence. Finally, a mechanistic understanding of how cigarette smoke inhibits endochondral ossification will be
explored, specifically whether a smoke-mediated inhibition of inflammation and chemotaxis results in the failure to produce the cartilaginous intermediate.
Appendix 1.1- Effects of cigarette smoke on preoperative levels of circulating osteoprogenitor cells. Cells are expressed as both relative percentages (above) and absolute number (below). Pre Fx – Pre-fracture, Smoked- animals receiving 28 days of daily smoke exposure, PBMNCs- Peripheral blood mononuclear cells.
Appendix 1.2 - Effects of cigarette smoke on early postoperative levels (postoperative day 4) of circulating osteoprogenitor cells. Cells are expressed as both relative percentages (above) and absolute number (below). Pre Fx – Pre-fracture, Smoked- animals receiving 28 days of daily smoke exposure, PBMNCs- Peripheral blood mononuclear cells.
Appendix 1.3 - Effects of cigarette smoke on postoperative levels (2 weeks postoperatively) of circulating osteoprogenitor cells. Cells are expressed as both relative percentages (above) and absolute number (below). Pre Fx – Pre-fracture, Smoked- animals receiving 28 days of daily smoke exposure, PBMNCs- Peripheral blood mononuclear cells.
Appendix 1.4- Effects of cigarette smoke on postoperative levels (3 weeks postoperatively) of circulating osteoprogenitor cells. Cells are expressed as both relative percentages (above) and absolute number (below). Pre Fx – Pre-fracture, Smoked- animals receiving 28 days of daily smoke exposure, PBMNCs- Peripheral blood mononuclear cells.
Appendix 1.5- Effects of cigarette smoke on postoperative levels (4 weeks postoperatively) of circulating osteoprogenitor cells. Cells are expressed as both relative percentages (above) and absolute number (below). Pre Fx – Pre-fracture, Smoked- animals receiving 28 days of daily smoke exposure, PBMNCs- Peripheral blood mononuclear cells.
Appendix 1.6- Effects of cigarette smoke on postoperative levels (5 weeks postoperatively) of circulating osteoprogenitor cells. Cells are expressed as both relative percentages (above) and absolute number (below). Pre Fx – Pre-fracture, Smoked- animals receiving 28 days of daily smoke exposure, PBMNCs- Peripheral blood mononuclear cells.
Appendix 1.7- Temporal changes in circulating osteoprogenitor cells following bilateral femur fracture surgery. Cells are expressed as both relative percentages (above) and absolute number (below). Fx – Fracture, OCN- Osteocalcin, SEM- Standard error of the mean.
Appendix 1.8- Temporal effects of cigarette smoke on circulating osteoprogenitor cells following bilateral femur fracture surgery. Cells are expressed as both relative percentages (above) and absolute number (below). Fx – Fracture, OCN- Osteocalcin, SEM- Standard error of the mean.
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