Impact of nicotine and PPARδ-agonist on human mesenchymal stem cells.

Samerna Bhat

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A Thesis
entitled
Impact of nicotine and PPARδ-agonist on human mesenchymal stem cells
by
Samerna Bhat
Submitted to the Graduate Faculty as partial fulfillment of the requirements for the
Master of Science in Biomedical Sciences Degree in Orthopaedic Sciences

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June, 2013
An Abstract of

Impact of nicotine and PPARδ-agonist on human mesenchymal stem cells

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May, 2013

Smoking is a well established factor in many diseases and has multiple systemic
effects due to more than 4000 different molecular species present in cigarette smoke.
Osteoporosis, low bone mineral density, increased nonunion and delayed union, and
increased risk of bone fracture have been associated with cigarette smoking. Nicotine, the
main component of cigarette smoke is responsible for addiction. Previous studies have
demonstrated deleterious impact of nicotine on skeletal remodeling and bone metabolism.
Although many studies in the past have used osteoblasts and osteoblast like cells to study
the impact of nicotine on bone, the use of human mesenchymal stem cells to determine
the effects of nicotine has been rare. Therefore, learning whether or not these cells that go
on to differentiate into osteoblasts and chondrocytes are also affected by nicotine will be
particularly valuable in predicting the prognosis of a smoker undergoing orthopedic
surgery/procedure. In this context, we examined the impact of nicotine in physiological
range (0.1µM to 10 µM) on: a) hMSC proliferation b) Calcium deposition by osteoblasts
(Alizarin red staining) c) Alkaline phosphatase activity (ALP assay on day) d) expression
of canonical genes during differentiation of hMSCs (western blot analysis). Our results
demonstrated a dose dependent decrease in hMSC proliferation, calcium deposition, ALP activity and expression of BMP-2 and HO-1. Interestingly, induction of heme oxygenase-1 (HO-1) by peroxisome proliferator-activated receptor delta (PPARδ) agonist, GW0742, prevented the negative effect of nicotine. These results led to the conclusion that nicotine has a damaging effect on hMSCs proliferation and osteogenic differentiation and the induction of HO-1 by GW0742 results in the reversal of these effects. This offers an opportunity for HO-1 inducers to be used as therapeutic agents to improve bone fusion and fracture healing in smokers and non-smokers.
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Chapter 1

Introduction

Cigarette smoking is one of the reversible leading causes of mortality and morbidity worldwide. In 2008, tobacco companies spent $10.5 billion or $29 million each day on marketing for both cigarette and smokeless tobacco.\(^1,^2\) United States happens to be the leading producer of tobacco leaves.\(^3\) Perhaps unsurprisingly, in 2010, it was estimated that close to 20% of adults in the United States smoke cigarettes.\(^4\) Annually, one in five deaths in the U.S. is related to smoking or tobacco usage.\(^5\) In fact, smoking-related deaths outnumber deaths caused by the human immunodeficiency virus, illegal substance abuse, alcohol use, motor accidents, suicides and murders combined! On an average, adults who smoke cigarettes die 14 years earlier than non-smokers.\(^6\) Many different cancers (bladder, cervix, kidney, larynx, pancreatic, stomach, etc.) are known to be caused by smoking. Lung diseases, coronary heart disease, peripheral vascular disease, chronic obstructive lung diseases are also caused by smoking. Furthermore, female smokers tend to have an increased risk for infertility, preterm delivery, stillbirth, etc.\(^7\)

Cigarette smoking has orthopaedic consequences as well. Both men and women smokers have an increased risk of hip fracture than non-smokers. Also, postmenopausal smokers have lower bone mass/mineral density compared with non-smokers. The probability of developing osteoporosis in women below the age of 65 increases if they are smokers.\(^8\) A negative effect on bone mass irrespective of weight is also associated with
smoking. In the military, female army recruits who developed stress fractures were more likely to be current or past smokers. Further, smoking and low back pain have been shown to be correlated in many studies. It has also been implicated in poor surgical outcomes, specifically when it comes to fracture healing. Indeed, surgeons urge stopping smoking a few weeks prior to a procedure in order to achieve shorter recovery times and stronger fusions post-surgery.

The mechanisms by which smoking can affect fracture healing are many and include reduced blood supply to the fracture site, increased blood level of reactive oxygen species (ROSs), decrease in anti-oxidant and vitamin levels and attenuating impact of nicotine on endothelial nitric oxide synthase.

As devastating health consequences as smoking may have, economic burden to the society is a heavy one. According to the Center for Disease Control, during 2000-2004, health-related economic cost to the society due to smoking was $193 billion, half of which was attributed to direct medical expenses and the other half to loss in productivity. In the United States alone, loss of 5.1 million years of potential life is attributed to smoking.

Given the level of seriousness of smoking-related impact on our society, it is imperative to understand how smoking contributes to poor bone health. However, it is important to understand cigarette smoke itself before its impact on bone can be understood and dealt with.
Cigarette smoke

There are two types of cigarette smoke: *mainstream smoke*, which is inhaled from the filter tip by the smoker and *sidestream smoke*, which is released into the environment from the burning end of the cigarette. Although both types of smoke possess highly reactive chemical species known as free radicals, it is the mainstream smoke which is abundant in two types of free radicals: reactive oxygen species (ROS) and reactive nitrogen species (RNS). In order to gain chemical stability, free radical species try to gain electrons from other nearby species. Once they do that, the neighboring species itself turns into a free radical causing a chain reaction. Cellular damage or death can be caused by both ROS and RNS through oxidative stress mechanisms.\(^{18}\) Depending on the brand and tar content, a person inhales approximately 2-3 mg of nicotine and 20-30 ml of carbon monoxide from each cigarette.\(^{18}\)

**Constituents**

Cigarette smoke contains more than 4000 potentially toxic constituents of which nicotine is the major component.\(^{19}\) Cigarette smoke exists in two phases: volatile phase (95% of cigarette smoke) consisting of volatile acids and particulate phase consisting of particulate matter. Carbon monoxide, carbon dioxide, ammonia, nitrogen, hydrogen cyanide and benzene are among the 500 different gases formed during volatile phase; and nicotine (C\(_{10}\)H\(_{14}\)N\(_2\)), anatabine (C\(_{10}\)H\(_{12}\)N\(_2\)) and anabasine (C\(_{10}\)H\(_{14}\)N\(_2\)) are among the 3500 chemicals produced during the particulate phase.\(^{20}\)

**Nicotine** is the key alkaloid in tobacco being 1.5% by weight in commercial cigarettes and amounting to about 95% of the total alkaloid content. Nicotine
Concentrations in oral snuff and pipe tobacco are similar to cigarette tobacco and those of cigar and chewing tobacco are only half of cigarette tobacco. Out of a total of 10 to 14 mg of nicotine in an average rod of tobacco, about 1 to 1.5 mg is absorbed systemically during smoking.\textsuperscript{21} Nicotine concentration in arterial blood can be as high as 100 ng/ml (usual range 20-60 ng/ml).\textsuperscript{21} During smoking, the plasma nicotine concentration ranges between 0.06 and 0.3 µM\textsuperscript{22} and its concentration in saliva of chronic snuff users may reach 0.6 to 9.6 µM.\textsuperscript{22}

**Mechanism of action of nicotine**

Nicotine is the addictive component of tobacco and is responsible for the central nervous system effects. Previously, nicotine has been shown to cause increased platelet aggregation, decreased prostacyclin level and inhibition of function of fibroblasts, red blood cells and macrophages. It exerts its effects by binding to nicotinic acetylcholine receptors causing an increase in blood level of catecholamines and formation of chalones. As a result, there is an increase in cardiac output and adrenergic vasoconstriction. Chalones interfere with the healing process by inhibiting epithelialization.\textsuperscript{21,23-26} By increasing platelet aggregation, nicotine causes sludging of blood and thus a generalized decrease in microvascular perfusion.\textsuperscript{27} A decrease in blood flow to the extremities as a result of nicotine induced peripheral vasoconstriction has also been demonstrated.\textsuperscript{28} A 29% decrease in blood flow to the hand was seen after smoking only two cigarettes.\textsuperscript{29} At cellular level, nicotine has deleterious effects on osteoblasts, macrophages and fibroblasts.
Absorption of nicotine

Nicotine inhaled from burning tobacco is first distilled and carried proximally on tar droplets. The extent of nicotine absorption across biological membranes is pH dependent. In acidic environment, nicotine (pKa 8) is present in ionized state and does not pass through membranes rapidly. Most cigarettes produce acidic smoke, which results in the predominance of ionized nicotine. As a result, there is small absorption of nicotine across buccal membrane. Other forms of tobacco (such as in pipes and cigars) produce a more alkaline smoke and as such, more unionized nicotine. There is considerable buccal absorption of nicotine from these tobacco products. Nicotine is rapidly absorbed through the small airways and alveoli of lungs because of their huge surface area. Additionally, the pH (7.4) of the fluids in the lung also contributes in the dissolution process and thereby transfer across membrane.\(^{30}\)

Distribution of nicotine in the body

When nicotine reaches the blood (pH 7.4) after absorption, it is about 31% unionized and 69% ionized. Liver, spleen, kidneys and lungs have the highest affinity for nicotine whereas adipose tissue has the lowest. Affinity of nicotine for brain tissue in smokers is greater than in non smokers because of the higher number of nicotinic cholinergic receptors in the former. Nicotine accumulates in saliva, gastric juice, breast milk, and also in fetal serum and amniotic fluid after crossing the placental barrier. The steady state volume of distribution of nicotine in body tissues averages 2.6 l/Kg. The pharmacologic effects after nicotine accumulates in the brain and other tissues depends on the route and rate of dosing. After smoking, nicotine rapidly reaches the pulmonary venous circulation.
followed by the left ventricle of the heart, the systemic circulation and finally the brain. It takes about 10-20 seconds to reach the brain. After smoking a cigarette, nicotine concentration in arterial blood can be as high as 100 ng/ml (usual range 20-60 ng/ml).

Impact of cigarette smoking on bone health

Cigarette smoking is correlated with many orthopaedic disorders (cf. Introduction). In order for scientists and clinicians to come up with solutions to solve those disorders, it is imperative to gain a basic understanding of what bone is (composition and physiology) and everyday processes like adaptation and healing of bone. The following sections aim to briefly cover the pertinent knowledge of the subjects aforementioned.

Bone composition

Bone is composed of 50 to 70% mineral, 20 to 40% organic matrix, 5 to 10% water and less than 3% lipids. Bone mineral is present as small crystals of needles, plates and rods arranged within or between collagen fibers and tends to be in the same direction as the collagen fibers. Hydroxyapatite \([\text{Ca}_{10}(\text{PO}_4)_{6}(\text{OH})_2]\) constitutes the majority of the mineral content, the rest being small amounts of carbonate, magnesium, citrate, fluoride and strontium. The load-bearing strength and mechanical rigidity of bone are due to bone mineral. The organic matrix is composed of 90% collagen and 10% non-collagenous proteins (osteocalcin, osteonectin, osteopontin and bone sialoprotein). Type I collagen is the predominant collagen present in bone matrix, the rest being trace amounts of types III and V and FACIT collagens (types IX, XII, XIV, XIX, XX, XXI). Collagen provides flexibility and tensile strength to bones. The role of noncollagenous protein has not been fully understood. Osteocalcin (Gla protein), the main noncollagenous protein is involved
in calcium binding, hydroxyapatite stabilization in bone matrix and regulation of bone formation. Gla protein has also been shown to inhibit premature mineralization.\textsuperscript{31-33}

**Types of bones**

**Cortical and cancellous bone**

The human skeleton is made up of 80% cortical bone and 20% cancellous bone. Cortical bone, also known as compact bone, is hard and dense and constitutes the outer part of all skeletal structures. Cancellous bone (or trabecular/spongy bone) is found in vertebrae, flat bones and the ends of long bones. It is composed of plate and rod-shaped trabeculae giving it a honeycomb appearance. As an example, in long bones, the diaphysis is composed mainly of cortical bone whereas the epiphysis and metaphysis are primarily made up of cancellous bone with a thin shell of cortical bone. The porosity of cancellous bone is 75% to 95% and that of cortical bone is approximately 5-10%. On a microstructural scale, the fundamental units of cortical bone and cancellous bone are referred to as an osteon (or Haversian system) and trabecular packet (or hemiosteon), respectively.\textsuperscript{31,32,34}

**Lamellar and woven bone**

Compact and trabecular bones, when visualized at a still finer scale of resolution reveal two more patterns: Lamellar and Woven. Lamellar bone is a highly organized bone which is formed slowly. Cortical and cancellous bones are mainly lamellar in pattern, where collagen fibrils are laid in alternating orientations within adjacent lamellae. Under polarized light, the laminations appear as alternating dark and light bands. Because of this alternating arrangement of collagen, the toughness of lamellar bone is significantly
increased. Woven bone on the other hand is formed quickly with poorly organized tissue. Collagen fibers and mineral crystals are laid in a disorganized random fashion in woven bone which makes it weaker than lamellar bone. Woven bone is normally produced at the time of primary bone formation and also in high bone turnover conditions like Paget's disease. It has a short life and eventually, gets replaced by lamellar bone. In humans, lamellar bone replaces woven bone in the long bones at age 2 or 3 years.\textsuperscript{31,32,34}

**Normal bone physiology**

Bone is a dynamic structure that undergoes continuous change to its structure and therefore, mechanical properties throughout life. Four different types of cells are involved in this continuous change:

**Osteoblasts**

These are derived from mesenchymal stem cells and are responsible for deposition of new bone. These cuboidal cells are mononuclear and are present on bone surfaces.\textsuperscript{35} Their main functions are: 1) Deposition of protein matrix, which will eventually form the organic matrix of bone and 2) Control of extracellular matrix mineralization. Osteoblasts communicate with each other via gap junctions and with osteoclasts by secreting factors like RANK-ligand (RANK-L), also called osteoclast differentiation factor (ODF). RANKL by binding to its receptor RANK, present on the surface of osteoclasts precursors and mature osteoclasts plays a key role in the recruitment, differentiation, activation and survival of osteoclastic cells.\textsuperscript{36} During matrix deposition, osteoblasts that get entombed within the matrix are called osteocytes.
Osteoclasts

These giant multinucleated cells are formed by the fusion of monocytes derived from hematopoietic cells. They can be as large as 100 mm in diameter. Their primary function is resorption of bone by secreting lysosomal enzymes like tartrate –resistant acid phosphatase, Cathepsin K and matrix metalloproteinases (MMPs) like collagenase. RANK-L secreted by osteoblasts activates the RANK receptor on osteoclast precursors. OPG is a secreted protein that binds RANKL with high affinity to inhibit its action on RANK receptor. Osteoclasts resorb bone by acidification and proteolysis of the bone matrix and the hydroxyapatite crystals. Completion of bone resorption is followed by apoptosis of osteoclasts.\textsuperscript{35,37}

Osteocytes

These are former osteoblasts which get embedded in the bone they deposit. Their functions include: 1) Functional adaptation of bone by detecting changes in the mechanical environment of bone (act as mechanosensory cells). 2) Bone turnover 3) Ion exchange through its large cell-matrix contact surface. Osteocytes sit in cavities called lacunae and communicate with each other and with osteoblasts through processes called canaliculi. Adjacent osteocytes communicate and exchange substances \textit{via} gap junctions.\textsuperscript{32,34,35,38}

Bone lining cells

These flat cells are also former osteoblasts (like osteocytes) and are present on the entire bone surface as a lining. Their functions include: 1) Releasing calcium when bone calcium is low. 2) Protecting bone from chemicals in blood. 3) Transporting mineral into
and out of bone. 4) Sensing mechanical strain. Bone lining cells communicate with osteocytes and with each other via gap junctions.32,35

**Adaptation**

Unlike man-made materials, bone is alive with activity; it adapts to its environment. For example, astronauts lose bone after prolonged space travel whereas tennis players have stronger bone in their arms. This fact has been known since the 19th century and is referred to as the Wolff's law (of adaptation). Continuous changes in bone, as suggested by Wolff's law are governed by two processes: Bone deposition and bone resorption. These two processes mediate how the bone tissue develops during normal growth or adapts to changes in the mechanical environment and disease, injury or surgical state. Adaptation occurs in one of the following three ways:

1) **Osteogenesis**

Osteogenesis occurs during embryonic development, early development and during fracture healing. It takes place in the following two ways:

**Endochondral ossification:** Vertebrae, ribs, long bones of the limbs, basal part of the skull and medial part of clavicles are formed by endochondral ossification. It is characterized by the presence of an intermediate stage of cartilage formation. It starts with the condensation of undifferentiated mesenchymal cells into shapes of future bones. This is followed by the differentiation of mesenchymal cells into chondrocytes, deposition of cartilage specific extracellular matrix, hypertrophy of chondrocytes and eventually, cell death by apoptosis. Simultaneously, osteoblasts originating from mesenchymal cells, along with blood vessels and osteoclasts, invade the zone of
hypertrophic chondrocytes. Osteoclasts break down the hypertrophic cartilage matrix and osteoblasts form a bone specific matrix utilizing the scaffold of degraded cartilage matrix. The bone matrix gets mineralized, which makes the skeleton strong.

**Intramembranous ossification:** Craniofacial bones and the lateral part of clavicle are formed by intramembranous ossification. It is characterized by the differentiation of mesenchymal condensations directly to osteoblasts without an intermediate stage of cartilage formation.

2) **Modeling**

During the modeling phase, bone deposition and resorption are uncoupled. This phase is prolonged and continuous and results in a change in size, shape or both. The pace of modeling decreases after skeletal maturity. As an example, to shape a bone, both modeling and remodeling are required to occur at different sites.32

3) **Remodeling**

During this phase, bone deposition and resorption are coupled. Remodeling is characterized by having a beginning and an end and not being prolonged like modeling. Usually no change in size and shape occurs. It is present throughout life.32

**Fracture healing**

Fracture healing can be of stable type or unstable type depending on callus formation. If callus formation is present before bone deposition, unstable fracture healing is said to have occurred. On the other hand, if there is no callus formation, fracture healing is said to be stable. Adequate blood supply is critical for both types of fracture repair.
**Unstable fracture healing**

Periosteum and marrow are two key tissues involved in fracture healing. Periosteum has two layers: an outer fibrous layer and inner layer known as cambium. Mesenchymal stem cells (MSCs) reside within the cambium layer of the periosteum. MSCs are also found in the endosteum, bone marrow and neighboring soft tissues. Unstable fracture healing is divided into the following types:

**A. Inflammatory phase**

The inflammatory phase lasts up to a week typically until bone or cartilage formation begins. Clinically, this phase is characterized by pain and swelling. There is serum exudation, hematoma formation and infiltration by inflammatory cells at the fracture site. Platelets are the first to reach the trauma site and release coagulation factors, TGF-β1 and platelet derived growth factor (PDGF). Recent evidence suggests that hematoma may act as a scaffold for the growing cells and its size may dictate the size of the callus. It is suggested that hematoma may induce the reparative stage of unstable fracture healing by releasing some growth factors. It is growth factors like the vascular endothelial growth factor (VEGF), which stimulate angiogenesis and bone formation. Blood vessel formation allows for the removal of necrotic bone and the formation of callus.³⁹
Figure 1 Stages of unstable fracture repair. Fracture repair begins with an initial inflammatory phase (up to a week) characterized by hematoma and severe inflammation. The next stage is marked by the occurrence of granulation tissue and is called the repair phase (up to a month). The final stage of fracture healing is the remodeling phase where the fibrocartilaginous callus gets mineralized and transformed into hard callus, which gradually disappears. (Adapted and modified from DJ G. Fracture healing. In: Ann L Johnson JEH, Rico Vannini, ed. AO Principles of Fracture Management in the Dog and Cat. 1st ed: Thieme Publishers; 2005.)

B. Reparative phase
This phase lasts up to a month and is characterized by the conversion of hematoma into granulation tissue. The maturation of the granulation tissue into connective tissue results in deposition of more collagen fibers. As the maturation progresses, types II and III collagen get replaced by type I collagen. Mesenchymal stem cells (from the endosteum, cambium layer of periosteum, bone marrow and adjacent tissues) which start proliferating during the inflammatory phase differentiate into chondrocytes during reparative phase. Transforming growth factor-β (TGF-β) and bone morphogenetic proteins (BMPs) play a key role in governing the chemotaxis, proliferation, coordination and differentiation of MSCs. External callus is formed by thickening of the periosteum around the fracture site and it gets its blood supply through extraosseous vessels. Internal callus (medullary callus) is formed inside the medullary canal from the endosteal cells and its vascular supply is through medullary arterioles. Together the external and the internal callus constitute the bridging callus, also called the soft callus. The external callus is strong enough to resist bending. Eventually, the soft callus becomes mineralized to form the hard callus.39

The reparative phase results in union of the fracture ends but the structure still differs from the original bone. At the end of this phase, bone is strong enough for a low impact exercise.

C. Remodeling phase:

This phase can last 6 to 9 years. The purpose of remodeling is to achieve optimal function and strength through a balance in bone resorption by osteoclasts and bone formation by osteoblasts. Over time, the external callus attains a more fusiform shape and is gradually
lost. Internal callus undergoes remodeling thus re-establishing the continuous medullary cavity. Fracture stabilization and a sufficient blood supply are the key factors in fracture healing.\textsuperscript{39}

**Stable fracture healing:**

There is no periosteal or endosteal callus formation in stable fracture healing and the fracture site is directly filled with bone. This type of healing is called primary healing. Compression plates or lag screws are used to attain stable interlocking of fracture fragments. This kind of precise fixation removes the biological signals which cause the osteoprogenitor cells from the neighboring tissues to travel to the fracture site and this eliminates the stage of callus formation.\textsuperscript{39}

Previous sections provided a brief overview of the fundamentals of bone composition, physiology, adaptation and healing. With this knowledge we will begin to have a meaningful understanding of bone-related disorders and cigarette smoking as a specific cause of those disorders. In the forthcoming sections, we will discuss the previous literature on the impact of smoking on bone and identify a crucial gap in our current understanding \textit{vis. a vis.} human mesenchymal stem cells.

**Mesenchymal stem cells (MSCs)**

MSCs are nonhaematopoietic multipotential cells that possess the ability to differentiate into both mesenchymal and non mesenchymal cell lineages. In 1966, Friedenstein and colleagues first described MSCs as adherent, colony forming, fibroblast like and spindle shaped cells, capable of differentiating into colonies resembling deposits of bone or
Mesenchymal stromal cells can be obtained from perivascular areas of various organs. They are capable of undergoing numerous cell divisions in vitro without losing their multipotency. They can differentiate into osteoblasts, adipocytes, chondrocytes, etc under specific stimuli. (Modified and adapted from Nombela-Arrieta CÅŠ, Ritz J, Silberstein LE. The elusive nature and function of mesenchymal stem cells. Nature Reviews Molecular Cell Biology. 2011;12(2):126-131.)

Figure 2 Mesenchymal stromal cells can be obtained from perivascular areas of various organs. They are capable of undergoing numerous cell divisions in vitro without losing their multipotency. They can differentiate into osteoblasts, adipocytes, chondrocytes, etc under specific stimuli. (Modified and adapted from Nombela-Arrieta CÅŠ, Ritz J, Silberstein LE. The elusive nature and function of mesenchymal stem cells. Nature Reviews Molecular Cell Biology. 2011;12(2):126-131.)

cartilage. These were then called as colony forming unit fibroblasts (CFU-Fs). CFU-Fs were shown to differentiate into adipocytes, osteoblasts, chondrocytes and myoblasts. MSCs have also been shown to differentiate into cardiomyocytes, neurons and astrocytes. Bone marrow is the principal source of MSC isolation. Other sources include adipose tissue, liver, muscle, amniotic fluid, placenta, umbilical cord blood and dental pulp. MSCs constitute only .001 to .01% of the total nucleated cells in the bone marrow. They are characterized by their ability to differentiate into multiple mature cell types under
specific stimuli. They possess extensive proliferative capacity in uncommitted state while retaining their multilineage potential. MSCs are positive for surface markers CD105, CD73, CD90, stro-1 and negative for hematopoietic cell surface markers like CD34, CD45, CD14, CD11a, CD19. These markers can however be expressed by other cell types as well. Till date, there is no single marker or a group of markers that is specific to MSCs.

When systemically transplanted in animals, MSCs migrate to site of injury and modulate the immune response through downregulation of proinflammatory cytokines and upregulation of prosurvival and anti-inflammatory factors. They inhibit T-cell proliferation \textit{in vitro} and modulate dendritic cell function by affecting their differentiation and maturation. They also interfere with the differentiation, cytotoxicity and cytokine secretion of natural killer cells. \textit{In vitro} osteogenic differentiation of MSCs can be induced by culturing the MSCs with dexamethasone (synthetic glucocorticoid), β glycerophosphate, ascorbic acid and 1, 25-dihydroxyvitamin D3. Preosteoblasts and mature osteoblasts can be detected by alkaline phosphatase activity and calcification of extracellular matrix respectively. Certain growth factors like insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) increase mineralization and alkaline phosphatase activity \textit{in vitro}.\textsuperscript{40-42}

Previous studies have demonstrated a positive impact of HO-1 inducers on the osteogenic differentiation of hMSCs.\textsuperscript{43,44} Based on those results, we used a PPAR\(\delta\) agonist (GW0742), which is an heme oxygenase 1 inducer, to study its impact on nicotine treated hMSCs. In the following section, a brief overview of Peroxisome proliferator-activated receptors and Heme oxygenase enzyme is provided.
Peroxisome proliferator-activated receptors (PPAR)

PPARs belong to nuclear hormone receptor superfamily and function as ligand activated transcription factors. They exist in three isoforms: PPARα, PPARβ/δ and PPARγ. According to the present nomenclature, PPARs are grouped in nuclear receptor 1C subfamily as NR1C1 (PPARα), NR1C2 (PPARβ/δ) and NR1C3 (PPARγ). Various compounds inducing peroxisome proliferation were identified in 1960s and onwards and were termed as peroxisome proliferators. In 1990, the receptor mediating peroxisome proliferation was identified in rodent hepatocytes, which was termed as PPARα. Shortly after, PPARβ/δ and PPARγ were characterized.

PPARs regulate a variety of cellular functions through isotype specific tissue expression. PPARα is expressed in tissues with high fatty acid catabolism like liver, heart, kidney, brown adipose tissue and intestine, where it regulates both mitochondrial and peroxisomal fatty acid oxidation by controlling the expression of vital enzymes. It plays an important role in the regulation of energy homeostasis by activating fatty acid catabolism and stimulating gluconeogenesis. PPARα activation leads to a decrease in circulating triglyceride level by causing increased fatty acid oxidation. This in turn lowers the lipid storage in liver, adipose tissue and muscle, thus resulting in improved insulin sensitivity. PPARα agonists are being used widely for treating dyslipidemias. PPARγ exists in two isoforms: 1) γ1, which is present in white and brown adipose tissues, intestine and immune cells and 2) γ2, expressed in white and brown adipose tissue. Its functions include adipocyte differentiation, lipid storage and glucose metabolism by causing improved insulin sensitivity. Thiazolidinediones and Glitazones which are synthetic PPARγ agonists are used to treat type 2 diabetes. Although PPARβ/δ
expression has been demonstrated in all tested tissues, it has been shown to be present in high levels in skeletal muscle, adipose tissue, skin, intestine, brain, inflammatory cells, heart and various types of cancer.\textsuperscript{45-47} PPARδ has numerous established roles in cardiac and skeletal muscle metabolism as well as in adipose tissue. It has been shown to have regulatory functions in wound healing, angiogenesis and adipose tissue formation. PPARδ activation has also been demonstrated to have anti-inflammatory and anti-atherogenic actions both directly as well as through heme oxygenase-1 (HO-1) induction by PPARδ agonists. HO-1 has also been shown to be involved in the regulation of osteoblast differentiation. Increased HO-1 expression decreases mesenchymal stem cell derived adipocytes but increases osteoblasts.\textsuperscript{43,44} Endogenous ligands for PPARβ/δ
include fatty acids, triglycerides, prostacyclins and retinoic acid. Although synthetic agonists like GW0742, GW2433, L-783483, L-165041 and GW501516 have been developed, they have not been used clinically as yet.

**Mechanism of action**

PPARs form heterodimers with retinoic X receptors (RXR) and upon ligand binding activate the transcription of their target genes depending on the presence of co-activators or co-repressors.

**Heme Oxygenase-1**

Heme oxygenase (HO) is present in all tissues and is located in microsomes and mitochondria. HO exists in two forms: HO-1 (inducible) and HO-2 (constitutive). It catalyses the rate limiting step in heme degradation resulting in the formation of carbon monoxide, iron and biliverdin. Biliverdin is rapidly converted to bilirubin by biliverdin reductase. HO-1 can be induced by numerous pharmacological agents as well as by conditions like cellular and oxidant stress, and heat shock. By degrading pro-oxidant heme to carbon monoxide (CO), bilirubin/biliverdin and free iron, heme oxygenase has been demonstrated to decrease the production of reactive oxygen species (ROS). Free heme, in excess, catalyses the formation of ROS that can result in endothelial cell dysfunction and various pathological conditions like hypertension and diabetes.\(^{48,49}\) The byproducts of heme degradation, bilirubin/biliverdin and CO possess anti-oxidant and anti-apoptotic properties respectively. Pharmacological agents that have been used to induce HO-1 include stannous chloride (SnCl\(_2\)), heme, heme arginate and Cobalt protoporphyrin (CoPP).
Figure 4 Functional consequences of the three heme degradation products, biliverdin, iron and carbon monoxide. Heme oxygenase catalyzes the degradation of heme to form biliverdin, carbon monoxide and iron. Biliverdin is subsequently converted to bilirubin by the action of biliverdin reductase. (Adapted from Cao J, Inoue K, Li X, Drummond G, Abraham NG. Physiological significance of heme oxygenase in hypertension. The international journal of biochemistry & cell biology. 2009;41(5):1025-1033.)
Chapter 2

Literature review

Bone formation and healing are mediated by a number of factors. In an in vitro study on rabbit osteoblasts, Ma et al.\textsuperscript{19} demonstrated that nicotine treatment resulted in a decrease in proliferation and expression of some key osteogenic mediators like BMP-2 and TGF-\textbeta. In a separate investigation\textsuperscript{50} on rabbits using a distraction osteogenesis model, they also demonstrated a decrease in mRNA expression of transforming growth factor \textbeta 1 (TGF-\textbeta 1), platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) in the nicotine treated group. Using a distraction osteogenesis rabbit model, another study was performed by the same group to evaluate the impact of nicotine on bone formation, angiogenesis and blood perfusion.\textsuperscript{51} Their results demonstrated a decrease in BMP-2 expression, significant decrease in blood perfusion, reduced bone volume (micro-CT analysis) in the distraction regenerate in the nicotine treated group in comparison to the control. Furthermore, histological examination showed a complete bony union in the distraction regenerate of the control group as opposed to mineralized matrix surrounding multiple loci of chondrocytes in the nicotine group. Interestingly, an increase in microvessel density was seen in the nicotine group in comparison to the control group. They concluded that nicotine had a direct inhibitory effect on osteoblasts by causing a decrease in BMP-2 expression. Although nicotine stimulates angiogenesis, the enhanced
angiogenesis cannot compensate for the ischemia due to nicotine induced vasoconstriction.

Another in vivo study by Theiss et al. showed that nicotine inhibited the expression of various cytokines involved in osteoblastic differentiation and neovascularization including collagen types I and II, BMP-2, 4 and 6, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). The authors used a rabbit model for spinal fusion.

Impact of nicotine on gene expression and osseointegration of titanium implants in rats was studied by Seiichi et al. They found that the bone implant contact ratio was lower in the nicotine treated group as compared to control group. In addition, a decreased expression of BMP-2, collagen type II, bone sialoprotein and osteopontin was also seen, suggesting that nicotine inhibits the genes involved in bone healing and as a result lessens osseointegration.

Previously, it has been shown that nicotine can have detrimental effects on bone metabolism and skeletal remodeling in vivo. Among patients having undergone hindfoot fusion procedure, smokers had significantly higher rate of non-union than non-smokers. The relative risk of developing a non-union was 2.7 times higher for smokers than for non-smokers.

A clinical study conducted on 85 patients undergoing treatment for lower leg fracture showed that current and previous smokers have 3 to 18 fold higher risk of delayed or nonunion than non-smokers. The percentage of delayed and non-union in smokers was found to be 14.1% and 10.6% respectively.
Rothem et al. demonstrated that nicotine had a biphasic effect on human osteosarcoma cells (MG63). Cells treated with nicotine concentrations (100 to 10,000 µM) similar to those present in a heavy smoker, demonstrated a decrease in both proliferation as well as downregulation of osteocalcin, alkaline phosphatase and collagen type I. On the other hand, nicotine concentrations similar to those present in a light to moderate smoker (0.01 to 10 µM) caused an increase in both the proliferation of osteoblasts as well as the expression of the investigated proteins. In another study by Rothem and co-workers on MG-63 (human osteoblast like cells), microarray analysis demonstrated that 100µM nicotine treatment caused changes in 842 genes. Gene ontology analysis revealed that nicotine altered various functions of MG-63 cells including developmental processes, cell-cell signaling, ion homeostasis, cell communication, intracellular signaling cascade, anatomical structure morphogenesis and response to external stimulus. Furthermore, a higher number of nicotine-altered genes with statistical significance were found in the 24 hour nicotine exposure group compared to the 5 hour exposure group.

Kim et al. used human alveolar bone marrow derived mesenchymal stem cells to study the impact of nicotine (1 µM to 5 mM) on survival, proliferation and differentiation of cells. Their results were in agreement with the study by Rothem et al. by demonstrating a bimodal effect but at different concentration range. Treating cells with nicotine concentration 1 µM to 100 µM did not have a significant impact on cell proliferation and differentiation. MTT assay demonstrated an increase in cell proliferation with 1 mM and 2 mM nicotine concentrations but a significant decrease with 5mM nicotine treatment. In comparison to control, alkaline phosphatase (ALP)
activity did not change with nicotine concentrations 1 µM to 1 mM but a significant decrease was seen at 2mM nicotine. Alizarin red staining for calcium deposition and expression levels of ALP, BSP, OCN, Runx2 and collα1 by PCR demonstrated the same pattern as that ALP assay. These differences in results might be due to different experimental conditions and different cell types used.

Schraufstatter et al. studied the effect of nicotine on hMSC apoptosis, migration and in vivo homing. They found out that nicotine concentration of $10^{-5}$ M caused cell death whereas no significant changes were found at nicotine concentration of $10^{-7}$ M. A decrease in C3a and bFGF mediated chemotaxis of hMSCs was also seen with $10^{-6}$ M nicotine treatment. Furthermore, nicotine ($10^{-9}$ M and $10^{-7}$ M) when injected into mice also caused a significant decrease in migration of hMSCs from peripheral blood to bone marrow and spleen.

Concurrent with our work, Ruiz and co-workers studied the impact of nicotine on the mechanical properties of mesenchymal stem cells. They used a similar concentration range of nicotine (0.1µM, 0.5 µM and 1 µM) as we have done in the present study (cf. Materials and Methods). Their results demonstrated that when compared to the control, the nuclear stiffness of the nicotine treated cells was significantly increased for all concentrations. The cytoplasm was significantly stiffer than the control for 0.5 µM and 1 µM nicotine concentrations. In previous studies, it has been shown that mechanical properties of stem cells play an important role in their ability to differentiate.
In a recent study by Ng et al., the impact of 1 µM nicotine on hMSCs and human periodontal ligament derived stem cells (PDLSC) was investigated.\textsuperscript{65} Cell proliferation in both hMSCs and PDLSC was significantly reduced in the nicotine treated group in comparison to the control group. When compared to control, more than 2-fold decrease in the total cell number was seen in both cell types at day 5 of nicotine treatment. Cell migration analysis on both hMSCs and PDLSC demonstrated a decrease in both the distance travelled as well as the speed of cell migration in the nicotine treated group. Furthermore, calcium deposition as well as alkaline phosphatase activity were also reduced in the nicotine treated group for both cell types. Microarray analysis of miRNA expression showed a dose dependent change in their expression from 0.5 µM to 1 µM nicotine concentration, suggesting that the negative effects of nicotine on stem cells might be caused by miRNA alterations. Finally, a downregulation of RUNX2, PTK2, ALPL, COL1A1, COL1A2 was seen in the nicotine treated group. These findings are in agreement with data collected from the present study.

Barbagallo et al. investigated the effect of HO-1 on MSC differentiation into osteoblasts. The authors used inducers of HO-1 expression and inhibitors of HO activity to assess the causality of the observed phenomenon. Their results demonstrated a 10.6 fold increase in HO-1 after 21 days of differentiation following CoPP (0.5 µM) treatment every 2 days. Furthermore, after treating hMSCs with 1 µM and 5 µM of CoPP in osteogenesis media for 14 days, alizarin red staining demonstrated a dose dependent increase ($p>0.05$) in calcium deposition in comparison to control. On the other hand, a decrease in adipogenesis was seen after treating hMSCs with CoPP (1 µM and 5 µM) in adipogenesis media for 14 days.
Figure 5 Scheme of the mechanism of HO-1 regulation of osteoblast and adipocyte differentiation. Increased HO-1 expression in MSCs caused an increase in osteogenesis by increasing BMP-2, Osteonectin, OPG and Osteocalcin (via increase in pAMPK and eNOS) Increased HO-1 shifts the balance of MSC differentiation in favor of osteoblast lineage and decrease in HO-1 or glucose exposure favors adipogenesis. (Adapted from Barbagallo I, Vanella A, Peterson SJ, et al. Overexpression of heme oxygenase-1 increases human osteoblast stem cell differentiation. Journal of bone and mineral metabolism. 2010;28(3):276-288.)

Their data reveal that CoPP-mediated increase in HO-1 results in a rise in MSC-derived osteoblasts and decreases adipocytes. An increase in osteoblast proliferation, differentiation and function was seen after CoPP treatment. In a follow-up study on hMSCs by the same group, osteogenic growth peptide (OGP) was used to induce HO-1 expression. It was found that OGP-mediated increase in HO-1 increases osteoblast...
proliferation, differentiation and function via an increase in AKT. Alkaline phosphatase expression and DNA levels showed a significant increase \( (p>0.05) \) in comparison to control following OGP treatment. On the other hand, HO-1 siRNA reversed the OGP mediated effect showing that OGP's positive effect on osteogenesis is caused by an increase in HO-1 expression. In the same study, it was demonstrated that CoPP (HO-1 inducer) treatment caused a significant increase in bone mineralization at day 21. These results are in corroboration with the results from our present study.

**Figure 6** Schematic diagram From: Heme oxygenase 1 regulates osteoclastogenesis and bone resorption. Increase in HO-1 expression downregulates RANKL receptor RANK, thus halting the further differentiation of osteoclast precursors to mature osteoclasts. (Adapted from Zwerina J, Tzima S, Hayer S, et al. Heme oxygenase 1 (HO-1) regulates osteoclastogenesis and bone resorption. The FASEB journal. 2005;19(14):2011-2013.)

Impact of HO-1 on osteoclastogenesis and bone resorption was studied by Jochen Zwerina et. al. They concluded that HO-1 induction by hemin inhibits osteoclastogenesis both in vitro and in vivo. Increased HO-1 expression resulted in
unresponsiveness of osteoclast precursors to M-CSF and RANKL by downregulating their respective receptors. Since this process is critical to osteoclast differentiation, further differentiation of osteoclast precursors to osteoclasts was arrested.

Previously, osteoblasts and osteoblast like cells have been used to study the impact of nicotine on bone but only a couple of studies have used human mesenchymal stem cells for evaluating the effect of nicotine on proliferation and osteogenic differentiation.⁴,⁵⁹

To conclude, our literature review led us to hypothesize the following:

1. Addition of nicotine will have a measurable negative impact on hMSC proliferation and osteogenic differentiation.

2. GW0742 will enhance osteogenesis, thereby ameliorating the negative impact of nicotine.
Chapter 3

Materials and methods

An overview of all experiments performed in this thesis is provided on the previous page (see Figure 7). The overview includes type of experiment, treatments and assay/staining procedures conducted. Frozen (in 10% DMSO) bone marrow mononuclear cells (Allcells, Emeryville, CA, USA) were thawed and resuspended in α-minimum essential medium (αMEM, Invitrogen, Carlsbad, CA, USA), supplemented with 20% heat inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and 1% antibiotic/antimycotic solution (Invitrogen, Carlsbad, CA). The cells were plated at a density of 1-5 x 10^6 cells per 75 cm² flask. The flasks were incubated at 37°C in a 5% CO₂ incubator and the medium was first changed after 48 hrs and thereafter every 3 days. After reaching approximately 70-75% confluence, the cells were recovered by the addition of 0.25% Trypsin/EDTA (Life Technologies, Frederick, MD) for the actual plating of the experiment. Specifically, culture media was removed from all the flasks and 4 ml of 0.25% Trypsin/EDTA was added to each flask. After about 5 minutes of incubation, the flasks were visualized under light microscope for the presence of floating detached cells. In case all the cells were not detached, the sides of the flasks were tapped to facilitate cell detachment. αMEM (4-5 ml) was added to the flasks to dilute and neutralize the Trypsin. The cell solution from each flask was transferred to a 15 ml centrifuge tube, which was centrifuged at 2000
**Figure 7** Schematic representation of the overall set of experiments, treatments and assays/staining. Passage 2-3 hMSCs were cultured in αMEM (MTT assay) or osteogenesis media (osteogenic differentiation experiments). MTT assay for cell proliferation was performed after 24 hrs and 7 days of nicotine treatment (alternate day) in physiological range. Alizarin red staining for mineralization was performed after 21 days of nicotine and GW0742 (PPARδ agonist) treatment. ALP staining and ALP assay were performed after 7 days of nicotine and nicotine plus GW0742 treatment respectively. Western blot was performed after 14 days of nicotine plus GW0742 treatment.
rpm for 3 minutes. The supernatant was carefully discarded without disturbing the cell pellet. αMEM (500 µl) was added to each tube to resuspend the cell pellet. After mixing well the cell solution, 10 µl of the homogenous cell sample was mixed with an equal volume of Trypan blue and the mixture was transferred to a hemocytometer. The total number of cells was calculated by counting the cells in 4 out of 9 chambers using a light microscope. Volume of the resuspended cell pellet to be added to αMEM (supplemented with 20% FBS) for different experiments was calculated from the total number of cells needed and the number of cells present per µl of cell solution (hemocytometer calculations). Media and cells were mixed well. Mesenchymal stem cells (passages 2-3) were cultured in a 75 cm² flask at a density of 1.5 million cells and cultured in alpha MEM supplemented with 20% FBS for 1 day at 37°C. Cells were treated with nicotine with or without GW0742 in αMEM or Osteogenesis media, the next day.

**MTT Assay for cell proliferation**

MSCs homogenously mixed in αMEM were plated in two 96 well plates. Specifically, MSCs were suspended in media at a density of 1 x 10⁴ cells/ml and 100µl of media was added per well (approx. 1000-1500 cells/well). Nicotine treatment and media change was carried out every alternate day to maintain nicotine concentration. The concentrations of nicotine used were 0 (cells in media without nicotine), 0.01 µM, 0.1 µM, 1 µM and 10 µM. Serial dilution of media with nicotine was done using 15 ml centrifuge tubes to get the desired concentrations of nicotine. After vortexing the tubes for a few seconds, the media plus nicotine solution (100µl) was transferred to the appropriate wells (with the adherent cells) of the 96 well plates. The number of samples in each group was 6. MTT assay was performed after 24 hrs (plate 1) and after 7 days (plate 2) of nicotine treatment.
This assay involves the conversion of water soluble yellow colored MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble formazan by metabolically active cells. After solubilizing, the concentration of formazan is determined by measuring the optical density at 570 nm. Vybrant MTT cell proliferation assay kit (V13154) was used according to the manufacturer's protocol. Specifically, the following steps were performed:

<table>
<thead>
<tr>
<th>Cells</th>
<th>Type of experiment</th>
<th>Treatment</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMSCs (Bone marrow)</td>
<td>Cell proliferation</td>
<td>0, 0.01, 0.1, 1, 10</td>
<td>MTT assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 hrs. 7 days</td>
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</table>

**Figure 8** MTT assay for cell proliferation and viability performed on hMSCs cultured in αMEM after 1) 24 hrs of nicotine treatment (treated once) and 2) 7 days of nicotine treatment (alternate day).

Sterile PBS (1 ml) was added to 5 mg vial of MTT to prepare a 12mM MTT stock solution. The solution was mixed well by vortexing. 10 ml of 0.01 M HCL was added to a 1 gm SDS in a tube and it was mixed well until SDS dissolved fully. Medium was replaced with 100 µl of fresh alpha MEM and 10 µl of the 12mM MTT stock solution was added per well. 10 µl of MTT stock solution added to 100 µl of medium alone was used as a negative control. The 96 well plate was then incubated for 4 hours at 37°C. Medium was carefully removed from the wells keeping only 25 µl in each well. DMSO
(50 µl) was added to each well and mixed properly. The plates were incubated at 37°C for 10 minutes. After mixing the samples again with a pipette, absorbance was read at 540 nm.

**Alizarin Red Staining**

**Figure 9** Alizarin red staining for calcium deposition was performed after 21 days of nicotine with or without GW0742 treatment (alternate day). hMSCs were cultured in osteogenesis media.

Human mesenchymal stem cells (hMSCs) were plated in two 24 well plates in osteogenesis medium at a density of 20,000 cells/cm² (Human/Mouse/Rat StemXVivo Osteogenic/Adipogenic Base Media (CCM007), R&D systems, Minneapolis, MN, USA) supplemented with Human StemXVivo Osteogenic supplement (catalog no. CCM008, R&D systems, Minneapolis, MN, USA) and were treated with nicotine (N) (Sigma Aldrich, St. Louis, MO, USA) with or without GW0742, a PPARδ agonist (Cayman chemical company, Ann Arbor, MI, USA), every alternate day. Plate 1: Control (cells in osteogenesis media without nicotine), 0.1 µM N, 1 µM N and 10 µM N. Plate 2: Control,
1 μM N, 10 μM N, 0.1 μM GW0742, 0.1 μM GW0742 plus 1 μM N and 0.1 μM GW0742 plus 10 μM N. The desired concentrations were obtained after serial dilutions. Alizarin red staining (EMD Millipore, Billerica, MA, USA) for calcium deposition was performed on day 21 according to the manufacturer’s protocol. Specifically, the following procedure were performed:

Media was carefully aspirated from each well without disturbing the cell monolayer. Cells were washed once with 2 ml PBS, then fixed with 10% formaldehyde and incubated at room temperature for 15 minutes. Formaldehyde was carefully aspirated and cells were washed thrice (5 minutes each time) with distilled water. After removing water, 1 ml Alizarin red solution was added to each well and the plates were incubated at room temperature for 40-45 minutes. Excess dye was removed and the cells were washed four times with deionized water with gentle shaking for 3-5 minutes. 1 ml water was added to each well to prevent the cells from dying.

The cells were then visualized using phase contrast microscope to determine the presence of mineralized nodules. Differentiated cells having mineral deposits stain bright red with alizarin red solution. Images were analyzed by Image-pro analyzer (Media Cybernetics, Inc., Bethesda, MD, ver. 6.2.0.424).

**Alkaline phosphatase (ALP) assay**

Cells were plated in 96 well plate in osteogenesis media at a density of 20,000 cells/cm² and were treated with nicotine (N) with or without GW0742 every alternate day along with media change for 7 days. The treatment groups chosen for this assay were: Control, 1 μM N, 10 μM N, 1 μM nicotine plus 0.1 μM GW0742 (PPARδ agonist), 10 μM N plus
0.1 μM GW0742 and 0.1 μM GW0742. Number of samples taken in each group (n) was 6. Alkaline phosphatase assay kit (Abcam, Cambridge, MA, USA) was used to measure ALP activity on day 8. ALP assay involves dephosphorylation of pNPP (p-nitrophenyl phosphate) to a yellow colored pNP (p-nitrophenol), the absorbance of which is measured at 405 nm.

The following steps were performed for the ALP assay: 5mM pNPP solution was prepared by adding 2 tablets of pNPP to 5.4 ml of assay buffer. The prepared solution was kept on ice and protected from light. ALP enzyme was reconstituted with 1 ml assay buffer.

**Protocol for Sample**

Sixty μl of supernatant from each sample was transferred into a new 96 well plate well. 50 μl of pNPP solution was added to each sample and mixed well. The plate was incubated at room temperature for 60 minutes, protected from light. Absorbance was read at 405 nm using microplate reader. Remaining supernatant from the original plate was discarded and 100 μl of assay buffer was added to each sample. The plate was incubated at room temperature for 10-15 minutes. The assay buffer from each group was collected in a 1.5 ml micro-centrifuge tube which was then centrifuged at 10,000 rpm for 1 minute. Sixty μl of the centrifuged supernatant was transferred to each well of a new 96 well plate. Fifty μl of pNPP solution was added to each well which was mixed well and then incubated at room temperature for 60 minutes, protected from light. To stop the reaction, 20 μl of stop solution was added to each well followed by gentle shaking. The absorbance was read at 405 nm using microplate reader.
Protocol for standard curve

Figure 10 Standard curve for ALP assay

Forty µl of 5mM pNPP solution was mixed with 160 µl of assay buffer to prepare 1mM pNPP standard. Then 0, 4, 8, 12, 16 and 20 µl of 1mM pNPP standard was added to a 96 well plate in duplicate to generate 0, 4, 8, 12, 16 and 20 nmol/well pNPP standard. The final volume was brought to 120 µl with assay buffer. This was followed by adding 10 µl of reconstituted ALP enzyme to each well. The ALP enzyme converts the pNPP substrate to an equal amount of colored p-nitrophenol (pNP). The plate was incubated at 25°C for 60 minutes, protected from light. The reaction was stopped by adding 20 µl of stop solution with gentle shaking. The absorbance was read at 405 nm using microplate reader.
Figure 11 ALP staining and ALP assay were performed after treating the cells with nicotine, and nicotine with or without GW0742 respectively for 7 days. Osteogenesis media was used for both the experiments.

**ALP Staining**

Human mesenchymal stem cells were cultured in 24 well plate in media for 7 days. Nicotine treatment of 0, 0.1, 1 and 10 µM was carried out every alternate day. Alkaline phosphatase staining (StemTAG alkaline phosphatase staining kit) was done on day 8 according to the manufacturer’s protocol. Specifically, the following steps were followed:

The medium was gently aspirated from the cells and the cells were washed with 1 ml of 1X PBS. PBS was then carefully aspirated. Fixing solution (0.4 ml per well) was added to the cells which were then incubated at room temperature for 2 minutes. After removing the fixing solution, the cells were washed twice with 1 ml of 1X PBS. PBS was aspirated and 0.4 ml of freshly prepared StemTAG AP staining solution was added per well. The cells were then incubated for 15-30 minutes at room temperature, protected
from light. After removing the staining solution, the stained cells were washed twice with 1 ml of 1X PBS.

**Western Blot**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Type of experiment</th>
<th>Treatment</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMSCs (Bone marrow)</td>
<td>Osteogenesis</td>
<td>0, 0.1, 1, 10, 1+GW0742, 10+GW0742, GW0742 14 days</td>
<td>Western blot (HO-1, ALP, osteopontin, BMP-2)</td>
</tr>
</tbody>
</table>

**Figure 12** Western Blot analysis of HO-1, BMP-2 and ALP was performed after alternate day treatment of hMSCs with nicotine and GW0742.

Cells were cultured in T 75 (75 cm²) flasks in osteogenesis media for 14 days at a density of 20,000 cells/cm² and treated with nicotine (N) with or without GW0742 and GW0742 alone every alternate day, along with media change. Each flask was for one group. Treatment groups selected for this experiment were: 1) Control (cells cultured in osteogenesis media without nicotine or GW0742), 0.1 µM N, 1 µM N, 10 µM N and 2) Control, 1 µM N, 10 µM N, 1 µM N + 0.1 µM GW0742 and 10 µM N + 0.1 µM GW0742 and 0.1 µM GW0742. On day 15, 0.25% Trypsin/EDTA was used to recover the cells for western blot analysis of BMP-2, HO-1, and ALP. The recovered cells were stored at -80°C until analysis. The following steps were followed for Western blot:
a) Homogenizing the samples

Samples were homogenized by adding 80-130 μl of homogenizing buffer (H.B) depending on the pellet size. Protease and phosphatase inhibitors were added to prevent the digestion of the sample by its own enzymes. The H.B volume used was approximately 5-6 times the pellet size. Samples were kept on ice for 30-45 minutes for the buffer to act and during that time, they were vortexed every 5-8 minutes for 5 seconds. In case sufficient protein was not present in the homogenate, samples were subjected to short pulses (1-3 seconds) of sonication to keep the samples from overheating. Cell samples were kept on ice throughout the procedure. This was followed by centrifugation of the homogenates @ 10,000 rpm for 10 minutes at 4° C. Supernatant was isolated and pellet was discarded.

b) Bradford protein assay

This assay is used to determine the concentration of protein in a sample. This involves the binding of Coomassie Brilliant Blue dye to proteins. Under acidic conditions, the dye is mainly in the doubly protonated red cationic form. When the dye binds to protein, it is converted into stable unprotonated blue form. It is this blue protein dye form that is detected at 595 nm using a spectrophotometer or a microplate reader. Predefined protocol from Bio-rad (Life science research, CA, USA) was followed. Specifically, the following steps were performed:

Bradford solution was prepared by adding 1 part of Bio-rad protein assay dye reagent concentrate (Bio-rad, CA, USA. Cat # 500-0006) to 4 parts of water. Being light sensitive, it was covered with aluminum foil. For each sample to be tested, 1 ml of the
prepared Bradford solution was taken in a cuvette in duplicate and 2 µl from the sample was added to it. The samples were kept on ice throughout the procedure. The solution was mixed well using a pipette. The presence of protein in the sample makes the solution change its color to blue. The protein concentration of the sample is directly proportional to the intensity of the blue color. The Bradford solution was allowed to act for 5 minutes before taking the readings using Eppendorf biophotometer (Eppendorf, NY, USA). Bradford solution with no sample was used as blank.

c) **SDS-PAGE (Sodium Dodecyl Sulphate Polyacralamide Gel Electrophoresis)**

Polyacralamide gels with 10 wells were prepared a day before running the samples and kept at 4°C overnight. Depending on the molecular weight of the protein of interest, 8% or 12% gels were made. The concentration of the acrylamide determines the resolution of the gel; high acrylamide concentration gels are used for smaller proteins and vice versa. Samples were mixed with water and loading buffer to get the final samples for loading. Their volumes were calculated according to the total protein concentration in individual samples (from Bradford protein assay), total volume (sample + L.B. + water = 22 µl) to be loaded per well and from total protein to be loaded in each well (30 µg). Before loading, the sample eppendorfs were heated at 90°C in water bath for 5 minutes to denature the proteins. This step allows opening up of the tertiary and secondary structures into primary structure, which makes it easy for proteins to run through the polyacralamide gel according to their molecular weights (kDa). Smaller and lighter proteins travel faster through the gel than heavier ones. The SDS-PAGE tank and gel chamber were filled with running buffer. Using loading tips, the first and the tenth wells of the gel were loaded with 7 µl of protein standards (Precision plus protein kaleidoscope
standards - 161-0375, Bio-Rad, CA, USA) and the rest eight with 22 µl of sample. A marker is a commercially available mixture of proteins, having defined molecular weights, typically stained so as to form visible colored bands. The gel electrophoresis was run at 90V till the separation of protein standards and then gradually increased to 120V. The SDS in the loading buffer covers the proteins with negative charge which then travel towards the positively charged electrode when voltage is applied. The blue line (Bromophenol in the loading buffer) helps keep track of the level the proteins are at. The gel electrophoresis was run till the blue line reached a little above the bottom of the gels.

d) **Transfer of proteins from gel to membrane**

Polyvinylidene difluoride (PVDF) membranes (Immobilon FL, EMD, Millipore corporation, Billerica, MA, USA), cut to the size of gels were immersed in methanol for 2-3 minutes, followed by immersing the membranes and blot papers (Bio-Rad, CA, USA) in transfer buffer. After completion of SDS-PAGE, gels were carefully separated from glass plates and placed on top of the membranes, which were then sandwiched between two blot papers. This sandwich was then placed on wet transfer cell (Transblot SD semidry transfer cell, Bio-Rad, CA, USA). Any bubbles formed were removed carefully. The membranes and the gel were kept wet throughout this step. The transfer cell was run at 140mA (0.14A) for 60 minutes.

e) **Blocking the membrane**

After the transfer of proteins to membranes, the membranes were put in blocking buffer for 1 hour on a shaker (Odyssey, Biocompare, CA, USA). Blocking buffer was diluted
with water (Blocking buffer: water = 1:4) before use. This step helps block any non-specific binding of antibody onto sites other than those which have the protein.

f) **Incubation with primary antibody**

After blocking, the membranes were cut according to the molecular weight of protein of interest and incubated with diluted antibodies. The dilutions (Antibody : Blocking buffer) used for different antibodies were: BMP-2 (ab14933, Abcam, Cambridge, MA, USA) - 1:700, HO-1 (ab13248, Abcam, Cambridge, MA, USA) - 1:1000, Alkaline phosphatase (ab95462, Abcam, Cambridge, MA, USA) - 1:1000, β-Actin (ab6276, Abcam, Cambridge, MA, USA) - 1:8000. The antibodies were incubated overnight at 4°C with gentle shaking.

g) **Incubation with secondary antibody**

Before placing the membranes in secondary antibodies, the membranes were washed thrice with Tris-buffered saline with Tween-20 (TBST) for 5 minutes each with gentle shaking. The dilutions (Antibody : Blocking buffer) used were : BMP-2 - 1:2000, HO-1 - 1:7000, Alkaline phosphatase - 1:3000, β-Actin - 1:10,000. This was followed by the incubation of the membranes with fluorescently labeled secondary antibody (Licor IRDye secondary antibodies, LI-COR Biosciences, US) solution for 1 hour at room temperature with gentle shaking. The secondary antibody boxes were wrapped with aluminum foil because of being light sensitive. After 1 hour the membranes were again washed with TBST thrice for 5 minutes each with gentle shaking.

h) **Detection**
Finally, the membranes were scanned using Odyssey infrared scanner.

**Data Reduction and Analysis**

All values were presented as mean ± S.D. and Student's *t*-test (two tailed, two sample with equal variance) was used to determine if the difference between groups was statistically significant. Difference between groups was considered significant at *p*<0.05. For western blot analysis, Image J software was used to compare the density (aka intensity) of bands.\textsuperscript{67}
Chapter 4

Results

MTT assay

A decrease in cell viability and proliferation was seen when exposed to nicotine in both the 24 hour as well as the 7 day group in comparison to control group. In the 24 hour treatment group, there was a significant decrease ($p < 0.05$) in cell survival at nicotine concentrations of 0.1 $\mu$M, 1 $\mu$M and 10 $\mu$M whereas the cells subjected to 7 day nicotine treatment demonstrated a statistically significant ($p < 0.05$) decrease in cell proliferation even at nicotine concentrations of 0.01 $\mu$M in addition to 0.1 $\mu$M, 1 $\mu$M and 10 $\mu$M nicotine.

Alizarin red (AR) staining

Plate 1: Alizarin red staining demonstrated a dose dependent decrease in mineralization after nicotine treatment of cells for 21 days. Plate 2: Here also, a dose dependent decrease in mineralization with increasing concentrations of nicotine was observed. Interestingly, the addition of 0.1 $\mu$M GW0742 to both 1 $\mu$M and 10 $\mu$M nicotine groups resulted in partial reversal of the inhibitory effects of nicotine. Phase contrast microscopy was used to determine the changes in cell morphology and presence of mineralized nodules. Captured images were analyzed using Image-pro analyzer (Ver. 6.2.0.424, Media Cybernetics, Inc. Bethesda, MD).
Figure 13 MTT assay performed for viability and proliferation of hMSCs demonstrated a statistically significant ($p<0.05$) decrease in proliferation for 0.1µM, 1µM and 10µM nicotine concentrations in the 24 hours treatment group. Nicotine treatment (alternate day) for 7 days caused a statistically significant ($p<0.05$) decrease in cell proliferation for all nicotine concentrations when compared to control.
Figure 14  Alizarin red and ALP staining. a) Alizarin red staining for calcium deposition was performed on day 21 of treating hMSCs with 0.1 µM, 1 µM, and 10 µM nicotine every alternate day. A dose dependent decrease in mineralization was demonstrated when compared to control. b) Alkaline phosphatase staining performed on day 8 of alternate day nicotine treatment (0.1 µM, 1 µM, and 10 µM) also demonstrated a dose dependent decrease in ALP staining. c) Alizarin red staining and alkaline phosphatase staining were quantified using Image-Pro Analyzer software.
Alizarin red staining for calcium deposition was carried out on day 21 of alternate day nicotine treatment (1µM and 10µM) with or without 0.1µM GW0742 and GW0742 alone. Both 1µM and 10µM nicotine treated cells showed a significant decrease ($p<0.05$) in mineralization when compared to control. Cells treated with 1µM nicotine + 0.1µM GW0742 and 10µM nicotine + 0.1µM GW0742 demonstrated a significant increase in mineralization when compared with 1 µM nicotine and 10µM nicotine respectively. Image Pro Analyzer was used to quantify the staining.
Figure 16 Alkaline phosphatase (ALP) assay for alkaline phosphatase activity was performed on day 8 of treating the cells with nicotine (alternate day) with or without GW0742 (PPARδ agonist) and GW0742 alone. The assay uses pNPP (p-nitrophenyl phosphate) which turns yellow when dephosphorylated by ALP produced by the differentiated cells. A statistically significant ($p<0.05$) decrease in ALP activity was seen with both 1µM and 10µM nicotine concentration. All the groups with GW0742 demonstrated a significant increase in ALP activity in comparison to control.

**ALP assay**

When compared to the control group (1.027), both 1 µM nicotine (0.948) and 10 µM nicotine (0.497) groups demonstrated a statistically significant ($p < 0.05$) decrease in alkaline phosphatase activity. Alkaline phosphatase activity values for groups 0.1 µM PPARδ agonist (GW0742), 1 µM nicotine plus 0.1 µM PPARδ agonist (GW0742) and 10 µM nicotine plus 0.1 µM PPARδ agonist (GW0742) were 1.283, 1.420 and 1.263 respectively, which were significantly ($p<0.05$) higher than the control group (1.027).

**ALP staining**

1.027
Alkaline phosphatase staining was performed on day 8 after treating hMSCs with 0, 0.1, 1 and 10 μM nicotine every alternate day. A dose dependent decrease in staining and hence alkaline phosphatase activity was seen.

**Western Blot**

![Western blot analysis](image.png)

Figure 17 Western blot analysis of HO-1 and BMP-2 was performed after treating the cells with nicotine (0.1 μM, 1 μM and 10 μM) in osteogenesis media every alternate day for 14 days. Quantitative densitometry analysis of bands was done using Image J software. Data are expressed as mean ± SD. p<0.05 was taken as significant.

Densitometry analysis of BMP-2/β-Actin for control, 0.1, 1 and 10 μM nicotine yielded 0.4163, 0.4182, 0.3738 and 0.3624 units respectively. A significant decrease in BMP-2 expression was seen in 1 μM and 10 μM nicotine groups in comparison to control. HO-1 also demonstrated a dose dependent decrease in expression with increased concentration of nicotine. An increase in both HO-1 and BMP-2 expression was observed when cells
were subjected to GW0742 treatment. For BMP-2, the densitometry values for control, 1 µM and 10 µM nicotine groups were 0.12, 0.15 and 0.16 respectively. In the same group, cells treated with GW0742, GW0742 plus 1 µM nicotine and GW0742 plus 10 µM nicotine yielded 0.840, 0.808 and 0.911 arbitrary units respectively. All three values were significantly higher than the control ($p > 0.05$). These same groups (GW0742, GW0742 plus 1 µM nicotine and GW0742 plus 10 µM nicotine) also showed significantly higher ($p > 0.05$) expression of BMP-2 than the 1 µM and 10 µM nicotine groups. For HO-1, the densitometry analysis of control, 1 µM and 10 µM nicotine yielded 0.2745, 0.2515 and 0.2125 respectively. There was a statistically significant ($p > 0.05$) decrease in HO-1 expression in 10 µM nicotine group when compared with control. Cells cultured in GW0742, GW0742 plus 1 µM nicotine and GW0742 plus 10 µM nicotine yielded a 0.49, 0.365 and 0.4535 arbitrary units for HO-1 respectively. These were all significantly higher ($p > 0.05$) than control, 1 µM nicotine and 10 µM nicotine group.
Western blot analysis of BMP-2 and HO-1 was performed after culturing hMSCs in osteogenesis media for 14 days. Treatment (alternate day) groups used were Control, 1 µM N, 10 µM N, 1 µM N + 0.1 µM GW0742, 10 µM N + 0.1 µM GW0742 and 0.1 µM GW0742. Image J software was used to perform the densitometry analysis of protein bands. Data are expressed as mean ± SD. *p<0.05 vs control was taken as significant.
Figure 19 Western blot analysis of alkaline phosphatase was performed after culturing the cells in osteogenesis media for 14 days. Treatment (alternate day) groups used were Control, 1 µM N, 10 µM N, 1 µM N + 0.1 µM GW0742, 10 µM N + 0.1 µM GW0742 and 0.1 µM GW0742. Image J software was used to perform the densitometry analysis of protein bands. Data are expressed as mean ± SD. *p < 0.05 vs. control; # p < 0.05 vs. 1; ^ p < 0.05 vs. 10; $ p < 0.05 vs. 1 + GW0742; @ p < 0.05 vs. 10 + GW0742.
Chapter 5

Discussion

Cigarette smoking is the leading cause of morbidity and mortality worldwide. In vitro, animal and human studies have demonstrated the damaging effects of smoking on bone. Smokers tend to have reduced fracture union and callus formation rates. Nonunion rates in human hindfoot fusion surgeries were found to be 2.7 times higher in smokers when compared to nonsmokers. Abstinence from smoking prior to surgery has been shown to improve post operative outcome. A beneficial outcome on wound healing has been seen in patients who did not smoke for five days before surgery. As a part of pre-operative preparation, orthopedic surgeons recommend cessation of smoking prior to surgery. Nicotine, a major component of cigarette smoke is responsible for addiction. Numerous studies have investigated the impact of cigarette smoking, smoke extract as well as nicotine alone on bone using human studies, animal models, osteoblasts or osteoblast like cells. Positive, negative and bi-modal effects were observed depending on the type of study, nicotine concentration, type of cell/animal model, route of nicotine treatment, duration of exposure and other experimental conditions. However, human bone marrow derived mesenchymal stem cells have rarely been used. Since it is MSCs that differentiate into osteoblasts, we chose these progenitor cells to investigate the impact of nicotine on osteogenesis.
The use of hMSCs in bone regenerative medicine has increased dramatically. Human mesenchymal stem cells have been used in fracture non-unions, vertebral disc degeneration, craniofacial defects, cartilage regeneration, tendon and ligament repair and other bone defects.\textsuperscript{69} When treated with hMSCs, long bone fracture nonunions demonstrated callous formation and graft integration after 2 months of surgery in comparison to 12 to 18 months with conventional treatment.\textsuperscript{70} However, numerous factors can influence the quality of hMSCs and cigarette smoking is one of the important ones, considering the huge number of people addicted to smoking. The quality of hMSCs for cell based therapies would be compromised if the donor or the recipient is a smoker. Increased levels of cigarette chemicals in smokers' blood could make the cells ineffective or less effective.

This study was conducted to investigate whether or not nicotine exposure has any deleterious effects on: 1) proliferation of hMSCs, 2) bone tissue mineralization, 3) alkaline phosphatase activity and 4) expression of canonical genes. Human mesenchymal stem cells treated with concentrations of nicotine (0.01 to 10 µM) within the physiological range, showed a dose dependent decrease in cell viability and proliferation, alkaline phosphatase activity, calcium accumulation and BMP-2, ALP and HO-1 expression when compared to the control group. During smoking, the plasma nicotine concentration ranges between 0.06 and 0.3 µM (Benowitz 1988) and its concentration in saliva of chronic snuff users may reach 0.6 to 9.6 µM (Hoover and Goldman 1992).

The proliferative capacity of mesenchymal stem cells is important because these cells migrate to the site of injury to facilitate and promote fracture healing by differentiating in to osteoblasts and, subsequently, depositing bone. The number and
function of osteoblasts is determined by growth factors like transforming growth factor β, basic fibroblast growth factor, insulin like growth factor, platelet derived growth factor and bone morphogenetic protein. BMP-2 was used in this study because of its osteoinductive property. BMPs are signaling molecules which play an important role in cell differentiation. They have a remarkable ability to induce cartilage and bone formation from non-skeletal mesenchymal cells. BMP-2 can direct these cells to commit to an osteoblastic pathway making it a great marker for osteoblast differentiation.

GW0742 (PPARδ agonist) was used in the present study based on the positive effect of HO-1 inducers from previous studies. In addition to functions like control over glucose and lipid metabolism, PPARδ also has a vasoprotective role against atherogenesis. It is in this latter role that PPARδ has been identified as an HO-1 inducer in the human vascular endothelium. There occurs an interaction between a co-activator PGC1α and PPARδ and HO-1 is induced through a ligand based signaling. The subsequent process occurs in a positive feedback loop fashion. The increased expression of HO-1 increases PPARδ promoter activity which results in increased expression of PPARδ target genes. The increase in HO-1 expression is crucial in determining the balance between osteoblastogenesis and adipogenesis in favor of osteoblast differentiation. Previous studies by Abraham et al. have demonstrated increased osteoblast differentiation resulting from over expression of HO-1. Both BMP-2 and HO-1 expressions demonstrated a dose dependent decrease with increasing concentration of nicotine. A similar trend was observed with proliferation and viability of cells, calcium deposition and alkaline phosphatase activity. Interestingly, the addition of PPARδ agonist restored all the functions of osteoblasts affected by nicotine.
The curative actions of PPARδ against the deleterious effects of nicotine are of clinical significance for normal rates of nonunion fractures ranging from 2.5 to 46%. If procedures like spinal fusion, degenerative spinal stenosis decompression and fusion are done on these patients, a disastrous nonunion outcome can result. There will be a drastic increase in medical costs because of additional procedures needed to correct nonunion, thus concomitantly decreasing the quality of life. Although, BMPs, autologous bone grafting, platelet rich plasma and other growth factors have been tried in patients with nonunion, no ideal method for treating this complication is yet available. Even though the iliac crest bone graft is regarded as the primary option for treating nonunions, there are limits to the amount of bone that can be harvested. Incidence of pain from the harvest site has been reported to occur in 37.9% patients and major complications including infection, large haematoma, prolonged wound drainage, reoperation and sensory loss has been reported in 8.6% cases. While an iliac crest bone graft was once considered as a prudent and economical option for the patient, the total cost for a 3 month perioperative period was recently estimated to average $37,227.

PPARδ agonists like GW0742 because of its healing effects on osteogenesis is a potential therapeutic agent to improve bone fusion and fracture healing. The restorative effects of GW0742 on osteogenesis through an increase in HO-1 expression can be utilized to improve bone fusion and fracture healing in smokers.
Chapter 6

Future work

Based on our results and the results from other in vitro, animal and human studies, it is advisable for smokers to stop smoking a few weeks before and after a procedure for the healing process to work optimally. It is important to point out that none of the studies mentioned establish the causality of the negative impact of nicotine on bone growth/healing. Nevertheless, there is an ever-growing body of evidence employing different models that suggests a strong inverse correlation between bone growth/healing and smoking, to which we must pay close attention for the benefit of the patients. On the other hand, several important questions must be answered before clinical relevance of this particular study is extrapolated to humans:

1. What is the impact of nicotine on cells such as osteoclasts, fibroblasts, epithelial and endothelial cells?

2. Under similar experimental conditions, how do hMSCs respond to nicotine vs. cigarette smoke extract?

3. How does the proliferative and osteogenic potential of MSCs from smokers, ex-smokers and non-smokers differ?
4. If in vitro studies suggested above provide consistent results, then animal *in vivo* studies using both nicotine and smoke extract must be carried out to assess clinical significance of HO-1 inducers for bone healing purposes.
References


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