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MESENCHYMAL STEM CELL DERIVED PROGENITOR CELLS

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Lay Abstract

There is concern that chemicals from oil sands mining in the Athabasca oil sands region are hurting the reproductive health of animals in the wild. Some of these animals, including bears, wolves, and river otters, need a bone in their penis called a baculum to reproduce. Studies have shown that some chemicals, including those from mining activity can make the baculum bone weaker. For bone to stay strong, bone cells always need to be developing to fix the bone tissue. The goal of our study was to find how chemicals from mining activity can affect the development of bone cells. We found that a group of chemicals that come from oil sands mining called naphthenic acid fraction components (NAFCs) stop bone cells from developing and making new bone. We also know that having more fat cells in bone is associated with weaker bones. We also looked at whether NAFCs could increase the development of fat cells. However, NAFCs did not increase the development of fat cells. Together, this research shows that NAFCs can make bones like the baculum weaker by slowing the development of new bone, but not by increasing fat cells. Our research suggests that exposure to NAFCs may make baculums weaker which may be bad for the reproductive health of animals living near oil sands mining activity.

Abstract

There is concern over the impact that petroleum related compounds (PRCs) associated with mining activity in the Athabasca Oil Sands Region (AOSR) are having on local wildlife. With the increase in oil sands mining activity in the AOSR there has been a corresponding decline in the fertility of indicator species in the AOSR. One of the primary sources of PRCs in the environment is oil sands process affected water (OSPW), which is stored in tailings ponds. Several PRCs, including naphthenic acid fraction components (NAFC), have endocrine disrupting effects, which may, in part, explain reduced fertility in indicator species. For example, male North American river otters (*Lontra canadensis*) living in areas impacted by mining activity have lower baculum strength those unaffected by mining activity. Weaker baculums are associated with increases in fracture rates and reduced fertility. Baculum strength is maintained throughout life by bone remodeling, a process that requires the differentiation of osteoblasts. NAFCs can impact several pathways integral to the development and path selection of mesenchymal stem cells into osteoblasts or adipocytes. Therefore, the objective of this thesis was to test the hypothesis that NAFCs inhibit osteoblast differentiation and induce adipocyte differentiation from progenitor cells. We exposed osteoblast progenitor cells and adipocyte progenitor cells to NAFCs. We demonstrated that NAFCs inhibit osteoblast differentiation and activate the glucocorticoid receptor pathway. We also found that NAFCs do not induce adipogenesis in adipocyte precursor cells. Lastly, we

showed that NAFCs are PPARγ ligands that inhibit the expression of PPARγ associated genes. These insights into the effects of NAFCs on osteoblast and adipocyte progenitor cells suggest NAFCs may contribute to lower baculum strength and impaired adipose tissue function of animals living in the AOSR. These effects my reduce the fertility and population of wildlife in the AOSR.

Publications during the acquisition of this degree

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List of all Abbreviations

ALP	Alkaline Phosphatase
aMEM	Alpha Minimum Essential Medium
AOSR	Athabasca Oil Sands Region
AP-1	Activator Protein 1
BCA	Bicinchoninic Acid Assay
BM	Bone Marrow
BMAT	Bone Marrow Adipose Tissue
BMD	Bone Mineral Density
BMP	Bone Morphogenic Protein
BSA	Bovine Serum Albumin
BTEX	Benzene, Toluene Ethylbenzene and Xylene
C/EBP	CCAAT/Enhancer Binding Protein
C/EBPβ	CCAAT Enhancer Binding Protein Beta
Cd36	Cluster of Differentiation 36
CNA	Commercial Naphthenic Acid
Col1a1	Collagen Type 1 alpha 1
CTL	Control
DMEM	Dulbecco's Modified Eagle Medium
ECL	Electrochemiluminescence
ECM	Extracellular Matrix
EDC	Endocrine Disrupting Chemical
EDTA	Ethylenediaminetetraacetic acid
FABP4	Fatty Acid Binding Protein 4
GR	Glucocorticoid Receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IBMX	3-isobutyl-1-methylxanthine
IGF-1	Insulin-like Growth Factor 1
IRF-3	Interferon Regulatory Transcription Factor 3
MSC	Mesenchymal Stem Cell
NA	Naphthenic Acid
NAFC	Naphthenic Acid Fraction Component
NF-kB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
OPG	Osteoprotegerin
OSPW	Oil Sands Process Affected Water

p85a	Phosphatidylinositol 3-kinase Regulatory Subunit Alpha
PAC	Polycyclic Aromatic Compound
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
PPIA	Peptidylprolyl Isomerase A
PRC	Petroleum Related Compound
Pref-1	Preadipocyte Factor-1
PTH	Parathyroid Hormone
PVDF	Polyvinylidene Difluoride
RUNX2	Runt Related Transcription Factor 2
SGK1	Serum/Glucocorticoid Regulated Kinase 1
STAT5	Signal Transducer and Activator of Transcription 5
Wnt	Wingless/integrated
Ywhaz	Tyrosine 3-monooxygenase/tryptophan 5-monooxygnase Activation Protein Zeta

Declaration of Academic Achievement

Robert Gutgesell (RMG) wrote the first draft of the thesis while Alison Holloway (ACH), Cheryl Quenneville, and Karen Beattie provided edits and feedback. In Chapter 1, ACH, Philippe Thomas (PJT), Jon Cormier and Sergio Raez-Villanueva were responsible for generating the data presented in Figure 1. In Chapter 2 RMG was the primary contributor to the data in Figure 1, Figure 2, Figure 3, and Figure 4. Laiba Jamshed (LJ) helped with some of the preliminary lab experiments. RMG wrote the first draft of the manuscript and the rest of the authors provided edits and feedback on the manuscript.

In Chapter 3, RMG was the primary contributor to the data in Figure 2, Figure 3, and Figure 4. LJ was the primary contributor to Figure 1. LJ and Raji Rajilingham helped with some of the preliminary lab experiments and cell culture. RMG wrote the first draft of the manuscript and the rest of the authors provided edits and feedback on the manuscript. For both Chapters 2 and 3 RMG, PJT and ACH were responsible for experimental design, and Mark Hewitt and Richard Frank provided the NAFC stock solution.

1. Chapter 1: Introduction

The Athabasca Oil Sands Region (AOSR) is one of the world's largest deposits of extractable bitumen. It is forecasted to contribute 1.7 trillion dollars to Canada's economy over the next decade (Doluweera et al., 2017). The extraction of bitumen requires a large amount of steam, producing 3-4 barrels of waste-water for every barrel of bitumen extracted (Canada, 2016). There are approximately 1.2 trillion litres of this wastewater stored in tailings ponds in the AOSR that needs to be isolated from natural ecosystems because of its contaminant load (Alberta Energy Regulator, 2019). There are four major categories of petroleum related contaminants (PRCs) in OSPW; naphthenic acids (NAs), polycyclic aromatic compounds (PACs), trace metals and BTEX (comprised of benzene, toluene ethylbenzene and xylene) (Huang et al., 2016). The primary routes of exposure for PRCs to the wildlife in the AOSR are seepage of oil-sands process affected water (OSPW) into natural watercourses, deposition into snowpack and atmospheric exposure to emissions from upgrading and other on-site industrial processes, and exposure to tailings ponds themselves (Fernie et al., 2018).

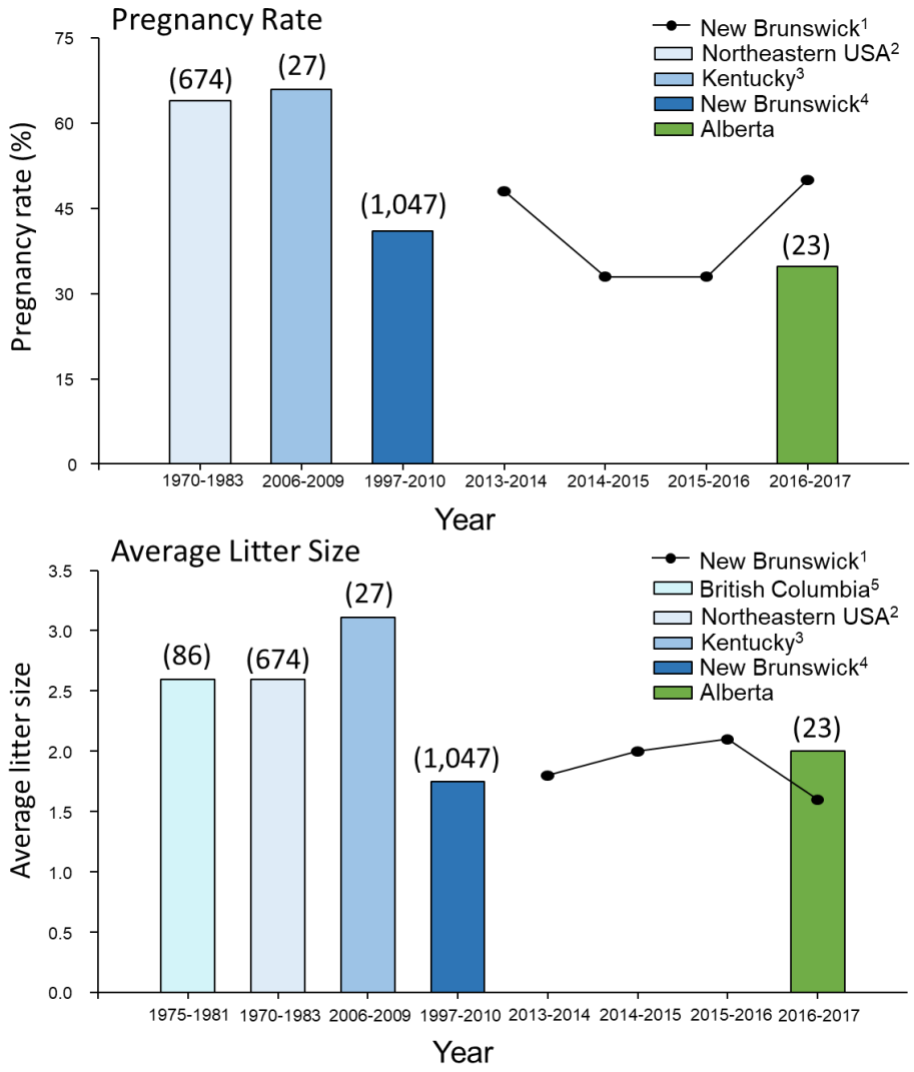

Figure 1. Comparison of measured fertility rate of otters in the AOSR and other locations in North America. Data for historical comparisons from **1**:(Government of New Brunswick, 2016), **2**:(Chilelli et al., 1996), **3**:(Barding EE, 2014), **4**:(Cumberland & DeVINK, 2017), **5**:(Stenson, 1975).

There is concern over the potential impact of PRCs on the local human and wildlife populations, and several PRCs have been associated with reduced fertility in mammals (Kassotis et al., 2016; Nagel et al., 2020; Sirotkin & Harrath, 2017). There is also evidence suggesting a decline in the reproductive rate of river

otters (*Lontra canadensis*) in the AOSR relative to historical data from other North American populations (Fig 1.)(Raez-Villanueva et al., 2019). North American river otters are a member of the Mustelidae family and are sensitive indicator species due to their high trophic position and semi-aquatic habitat that makes them susceptible to contaminants in the rivers and lakes in the AOSR in addition to soil and air-based contaminants. Thomas et al. (2021) investigated whether proximity to oil sands mining activity in the AOSR influenced the baculum strength of river otters (*Lontra canadensis)*. The baculum is a mineralized long bone found in the glans penis in many mammal species, and while its function is not entirely understood, it is thought to provide structural support during mating (Brassey et al., 2018). Thomas et al. found that river otters trapped in areas with high levels of mining activity had baculums that were less stiff and failed at a lower peak load than those trapped in areas within the AOSR that were unaffected by mining activity (Thomas et al., 2021). Bone strength can be affected by a variety of endocrine factors, including sex hormones and glucocorticoids that impact different parts of remodeling (Canalis & Delany, 2002; Khosla et al., 2012; Mohamad et al., 2016). Exposure to endocrine disrupting chemicals (EDC) has been shown to be associated with lower baculum bone mineral density (BMD) and increased likelihood of baculum fracture; effects that may impair reproductive success (Sonne et al., 2015). Thus, the decreased baculum strength observed by Thomas et al. may be a contributing factor in the low fertility rate observed in the river otters in the AOSR (Raez-Villanueva et al.,

2019). Several PRCs have endocrine disrupting effects, including some PACs that have been found to have steroidogenic effects (S. Lee et al., 2017), and several metals that have been reported to have estrogenic effects (Paschoalini et al., 2019; Yang et al., 2015). In addition, NAs extracted from OSPW can act as androgen and estrogen receptor antagonists (Leclair et al., 2015). Androgen and estrogen are involved in regulating bone remodeling by acting on both osteoblastogenesis and osteoclastosgenesis, and are required to maintain bone mass (Vanderschueren et al., 2004). Therefore, it is plausible that PRCs are contributing to the lower bone strength phenotype observed in animals living in close proximity to active mining sites in the AOSR.

1.1.1. Naphthenic Acid Fraction Components

NAs are present in all crude oil and are concentrated in OSPW as a by-product of bitumen extraction (Wu et al., 2019). Nonpolar organics including NAs are considered to be responsible for the majority of the toxic effects of OSPW and for this reason they are among the most studied PRCs in OSPW. However, the majority of these toxicology studies have used technical NA mixtures that are available commercially. NAs are classically defined as $C_nH_{2n+Z}O_2$, where Z indicates hydrogen atoms lost to ring formation, and commercially available NA mixtures are comprised of classical NAs. The naphthenic acid fraction components (NAFCs) of OSPW differ from commercial mixtures in that they are more complex than NAs with higher proportions of ring

structures and oxygen species (Bauer et al., 2019; Brunswick et al., 2017; Marentette et al., 2015; Vander Meulen, Schock, et al., 2021). Consequently, commercial NAs and NAFCs may have different toxicologic effects in some tissues (Bartlett et al., 2017; Loughery et al., 2019).

NAFCs are present in surface and ground water samples in the AOSR because bitumen is naturally present in rock formations and riverbeds, however, these concentrations are generally below 1mg/L. Wetlands near AOSR mining have reported concentrations between 8.1 and 36.1mg/L, whereas OSPW contains concentrations of NAFCs up to 110mg/L (Headley & McMartin, 2004; Vander Meulen, Klemish, et al., 2021; Vander Meulen, Schock, et al., 2021). However, these concentrations vary greatly depending on the measuring technique and there is not a standard and robust method of measuring NAFC concentrations (Brown & Ulrich, 2015). While the exact exposures of wildlife to NAFCs cannot be measured at this point, researchers have in some cases been able to use novel chemical markers to confirm that OSPW from tailings ponds have migrated into groundwater reaching the Athabasca River system (Hewitt et al., 2020). This evidence of seepage of OSPW into natural waterways in the AOSR suggest that aquatic animals are likely being exposed to greater quantities of NAFCs than appear in waterways from natural bitumen due to oil sands activity.

NAFCs and OSPW have been reported to have adverse effects on the reproductive health of animals. In one study treatment of Sprague Dawley rats with commercial naphthenic acids at doses of 300 and 900mg/kg/d significantly

reduced the number of live born offspring (McKee et al., 2014). Similarly, female Wistar rats that were given naphthenic acids isolated from AOSR OSPW (60 mg/kg/d, 5 days per week orally) had some reduced fertility but no malformations among offspring (Rogers et al., 2002). Other effects reported by Rogers et al. in these female rats were increased liver weights, elevated blood amylase and hypocholesterolemia and excessive hepatic glycogen accumulation (Rogers et al., 2002). In contrast to mammalian studies, a number of studies have investigated the effects of NA and OSPW on reproductive outcomes in fish species; reported adverse effects include reduced fertilization success, premature hatching, embryo deformities, and reduced expression of secondary sexual characteristics (He et al., 2012; Kavanagh et al., 2013; Li et al., 2017; Peters et al., 2007). In addition, larvae exposed to NAFC had skeletal deformities (Loughery et al., 2019) and OSPW has been shown to affect adipogenesis (Peng et al., 2016). Both osteoblasts (bone cells) and adipocytes (fat cells) share a common mesenchymal cell lineage. As bone strength can be affected by both the development of bone and the interface between bone and bone marrow adipose tissue (BMAT), any exposure that can affect the differentiation of these mesenchymal precursor cells into osteoblasts or adipocytes has the potential to influence bone strength. However, the effects of NAFCs from OSPW on osteoblastogenesis or adipogenesis are unknown.

1.1.2. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are pluripotent fibroblast-type stem cells that can differentiate into osteoblasts, adipocytes, chondrocytes, myocytes, and neurons (Pittenger et al., 1999). MSCs were first found in bone marrow in 1976 (Friedenstein et al., 1976) and since have been found in human umbilical cord (Wang et al., 2004), placenta (Antoniadou & David, 2016), adipose tissue (Casteilla et al., 2005) and in dental pulp (Leong et al., 2012). It is now well known that chemicals can influence MSC lineage commitment *in vivo* (Bateman et al., 2017). For example, treatment with rosiglitazone, a PPARγ agonist, induces the differentiation of MSCs in bone into adipocytes and suppresses osteoblastogenesis resulting in an increase in bone marrow adiposity and reduced bone strength and quality (Ali et al., 2005). Bone marrow adiposity is associated with reduced bone quality and has been observed in most bone loss conditions (Bredella et al., 2011; Moerman et al., 2004). Differentiation path selection is determined by the biological, chemical and physical milieux surrounding the MSCs. These factors converge to affect transcription factors driving the differentiation of MSCs toward adipogenesis (C/EBPs and PPARγ) or osteoblastogenesis (RUNX2 and TAZ) (Q. Chen et al., 2016).

1.1.3. Bone

There are five types of bone: long, short, flat, irregular, and sesamoid. The baculum is a long bone; long bones are typically longer than they are wide and have a rounded head (epiphysis) at both ends and a long shaft (diaphysis) that

carries the load between the epiphyses. The diaphysis is typically loaded as a simply supported beam; its mechanical strength is a product of the properties of the material and its cross-sectional area.

Bone is a composite material made of mineral crystals bound to proteins. This construction combines the strength and resilience of the proteins with the stiffness and hardness characteristics of mineral. The mineral phase is comprised of crystals containing calcium and phosphate, called hydroxyapatite, and the protein phase consists of collagen (Glimcher, 2006). The collagen is assembled into fibers, and along with other proteins these are bound to the mineral crystals (Glimcher, 2006). To provide an optimal stiffness to mass ratio, bones are hollow with the dense outer cortical shell farthest from the neutral axis. The hollow interior contains a fine network of trabecular bone while the outer cortical shell provides the mechanical strength and attachment sites for tendons (Clarke, 2008). Bone strength is a product of BMD and matrix integrity and is maintained through the continuous process of remodeling. Bone remodeling involves the resorption of old bone matrix by osteoclasts and the deposition of new bone matrix by osteoblasts. This process both repairs microdamage to the bone and allows the bone to adapt to novel loading stimuli. The relative rate of deposition to resorption determines the rate of change of BMD (General (US), 2004).

Bone tissue is made up of three major cell types: osteoblasts, osteocytes, and osteoclasts. Osteoblasts are located on the osteoid and synthesize new bone matrix on bone-forming surfaces as they differentiate from osteoblast progenitor

cells during the formation phase of the remodelling cycle. Osteoprogenitor cells are derived from multipotent mesenchymal stem cells (MSCs), which can also differentiate into adipocytes, chondrocytes, myocytes, and neurons. As the cells age, 60-80% of mature osteoblasts undergo apoptosis while the rest become embedded in the matrix and differentiate into osteocytes (Komori, 2016). Osteocytes are terminally differentiated osteoblasts and are embedded in the bone matrix. Osteocytes are the most plentiful cell type in bone and have mechanosensory and regulatory function; they can emit stress signals in response to changes in bone strain imposed by mechanical loading and react to endocrine signaling to regulate bone remodelling (K. Lee et al., 2003). Osteoclasts are multi-nucleated cells that perform the resorption phase of bone remodeling and are derived from hematopoietic mononuclear precursor cells of the monocyte-macrophage lineage (Clarke, 2008). Osteoclast activation is dependent on osteocyte and osteoblast release of receptor activator of NF-κB ligand (RANKL) and macrophage colony stimulating factor (M-CSF)(Rauch et al., 2010). Once active, osteoclasts dissolve the mineral content of the bone by secreting H+ ions and secrete the cathepsin K enzyme that digests the protein content of the matrix (Komori, 2018). Bone remodeling is regulated by communication between osteocyte, osteoblast and osteoclast cells and controlled by local and systemic factors to fulfill both the structural and metabolic functions of bone (Hauge et al., 2001).

Initiation of remodeling is caused by signaling from osteocytes and begins with the activation phase. In targeted remodeling, osteocyte apoptosis leads to release of paracrine factors that increase local angiogenesis and recruitment of osteoclast and osteoblast precursors (Atkins & Findlay, 2012; H. Chen et al., 2015; Dallas et al., 2013; Tatsumi et al., 2007). Non-targeted remodeling occurs in response to hormones, allowing access to bone calcium stores, not directed to a specific site (Kenkre & Bassett, 2018). Next, during resorption osteoclasts pump protons generated by carbonic anhydrase II onto the bone surface being resorbed, dissolving the bone mineral (Kenkre & Bassett, 2018). The remaining collagen is degraded by proteases including cathepsin K and matrix metalloproteinases. Resorption ends by osteoclast programmed cell death (Kenkre & Bassett, 2018). The reversal phase follows resorption in which freshly resorbed bone surface is prepared for deposition in new bone matrix by osteoblastic cells that remove unmineralized collagen matrix. Signaling between osteoblasts and osteoclasts occurs to ensure no net bone loss (Howard et al., 1981; Sims & Martin, 2015). Osteoclasts express ephrinB2 and osteoblasts express EphB4 ephrin receptor, suggesting that the reversal phase may be controlled by bidirectional ephrin signaling from osteoblasts to osteoclasts (Zhao et al., 2006). Other signaling pathways including BMP-2, TGF-B and IGF-1 may also be involved (Sims & Martin, 2015; Zhao et al., 2006). Bone formation occurs in two steps: first osteoblasts synthesize and secrete a type 1 collagen-rich osteoid matrix, then osteoblasts play a key role in regulating osteoid mineralization (Atkins & Findlay,

2012). After mineralization is complete, osteoblasts undergo apoptosis, become bone-lining cells or terminally differentiate into osteocytes embedded in the bone matrix.

Few studies have investigated the effect of NAs or OSPW exposure on bone, and no studies have investigated the effect of NAs on bone metabolism, osteoclastic or osteoblastic activities. One study that reported on the effects of naphthenic acid fraction components (NAFC) from aged or fresh OSPW and commercial NAs on fathead minnow larvae reported bone deformities and altered transcriptomic networks underlying skeletal development. Skeletal gene networks of chondrocyte development and endochondral ossification were altered by NAFC and commercial NA. Spinal deformities were most pronounced in larvae exposed to aged NAFC, and transcriptomic profiles were more similar between fresh and aged NAFC than commercial NA (Loughery et al., 2019). In another study, exposure to the water-soluble fraction of light cycle oil that contains several PRCs resulted in lower bone mineralization in sea bass (Danion et al., 2011). Several groups have reported on the effects of NAs or OSPW on hormones that can affect bone strength. For example, exposure to 10 mg/L naphthenic acids extracted from discharge water from Statoil platform Oseberg C did not change cortisol production, but high doses (10µg dry weight $m^{-1}L^{-1}$) did increase 17B-estradiol and progesterone production and decrease testosterone production in human adrenocortical carcinoma (H295R)-cells, but lower doses (0.1 and 1µg dry weight $m^{-1}L^{-1}$) did not (Knag et al., 2013). Both estrogen and testosterone are important

for maintenance of bone health, and a decrease in testosterone in particular may result in lower bone strength (Khosla et al., 2012; Mohamad et al., 2016). Although Lister et al. (2008) found that goldfish exposed to OSPW but not naphthenic acid extract had increased plasma cortisol levels, which they suggest indicates an effect on glucocorticoid hormone biosynthesis, the serum cortisol data for the naphthenic acid extract treated goldfish was not presented in the paper (Lister et al., 2008). These studies suggest several potential mechanisms by which OSPW or NAFCs may inhibit bone strength, although little is known of their direct effect on MSCs or osteoblasts.

1.1.4. Bone marrow adipose tissue

Bone marrow adipose tissue (BMAT) comprises about 70% of bone marrow (BM) volume (Cawthorn et al., 2014) and is functionally distinct from the other two primary types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT), in that it resists insulin- and cold-stimulated glucose uptake (Suchacki et al., 2020). There are two types of adipocyte populations that comprise BMAT: regulated BM adipocytes that are interspersed with haemopoietic BM and constitutive bone marrow adipocytes that cluster together at distal skeletal sites (Bukowska et al., 2018). The majority of BM adipocytes arise from leptin-receptor positive MSCs, a quiescent progenitor cell population that can differentiate primarily into either mature adipocytes or osteoblasts (Zhou et al., 2014). Marrow adiposity is correlated with low bone mass and the fate

decision of MSC progenitor cells into adipocytes or osteoblasts appears to be dependent on peroxisome proliferator-activated receptor gamma (PPARγ) activity; PPARγ insufficiency increases bone formation and PPARγ overexpression inhibits it (Akune et al., 2004; S. W. Cho et al., 2011). The primary regulatory transcription factors of adipogenesis and osteoblastogenesis, PPARγ and runt-related transcription factor 2 (RUNX2), are reciprocally regulated BM MSCs (Stechschulte & Lecka-Czernik, 2017).

As with bone, few studies have reported on the effects of NAFCs or OSPW on adipocytes. One notable exception is a study by Peng et al. (2016) in which they identified components of OSPW as PPARγ ligands and were able to induce PPARγ-mediated adipogenesis in murine 3T3-L1 adipocyte progenitor cells treated with OSPW (Peng et al., 2016). Peng et al. also investigated the effects of fractionated OSPW and found that while the nonpolar fraction, which contained most NAs, did contain PPARγ ligands, it did not induce adipogenesis to a similar degree as the other fractions of OSPW (Peng et al., 2016). The activation of PPARγ by other environmental contaminants has been shown to lead to the induction of adiposity in animals (Fang et al., 2015; Feige et al., 2007; Harada et al., 2015; Pillai et al., 2014). In fact, a study that investigated the effect of exposure to the wastewater from fracking, another oil extraction method, found an induction of PPARγ -mediated adipogenesis (Kassotis et al., 2018). NAFCs also inhibit mitochondrial respiration, uncouple oxidative phosphorylation and increase reactive oxygen species emission in isolated mitochondria harvested

from trout liver (Rundle et al., 2018). This impaired mitochondrial function could affect oxidative metabolism in adipocytes, impairing their ability to generate energy to support important metabolic pathways like triglyceride synthesis and gluconeogenesis. Taken together these studies suggest that NAFCs may have an impact on adipocyte function as well as differentiation.

1.1.5. Pathways regulating MSC differentiation

The differentiation of MSCs into mature osteoblasts or adipocytes is regulated by many signaling pathways at different stages of development, but the focus of this section are two pathways that have substantial impacts on MSC differentiation and are likely targets for modulation by exogenous chemicals. Exogenous glucocorticoids are well known to induce a loss in BMD (Briot & Roux, 2015) and many endocrine disrupting chemicals affect either serum cortisol levels or act via the glucocorticoid or mineralocorticoid receptors (Diamanti-Kandarakis et al., 2009; Zhang et al., 2019). Secondly, as discussed above, activation of PPARγ is capable of inducing differentiation of osteoblast progenitor cells to an adipocyte phenotype (E.-S. Cho et al., 2012). Importantly OSPW has previously been found to contain PPARγ ligands (Peng et al., 2016; Stechschulte & Lecka-Czernik, 2017). Therefore, it is biologically plausible that if NAFC can activate either the glucocorticoid receptor (GR) or PPARγ pathways in MSCs there could be an inhibition of osteoblastogenesis or an increase in bone marrow adipose tissue; both of which have the potential to impair overall bone strength.

Glucocorticoids have adverse effects on skeletal health, and glucocorticoid-induced osteoporosis is the most common form of secondary osteoporosis(Briot & Roux, 2015). Secondary osteoporosis is the loss of bone mineral density from any clinical condition other than aging (Briot & Roux, 2015). Glucocorticoids exert their effect on the skeletal system through both direct effects on bone and indirectly by modulating the neuroendocrine system, calcium metabolism and inducing muscle myopathy (Canalis et al., 2007). Glucocorticoids have direct effects on each of the three major bone cell types, inhibiting differentiation and function and inducing apoptosis in osteoblasts, inhibiting the function and inducing apoptosis in osteocytes, and by increasing osteoclastogenesis and reducing osteoclast apoptosis (Canalis et al., 2007). Although low levels of GR activity induced by endogenous glucocorticoids is required for bone formation (Sher et al., 2004), exogenous glucocorticoids reduce both bone mass and quality through reduced bone formation and increased resorption. Glucocorticoids inhibit osteoblastogenesis by regulating the nuclear factors of the CCAAT/enhancer binding protein (C/EBP) family and the peroxisome proliferator activated receptor gamma 2 (PPARγ2) and inhibiting the transcriptional repression of preadipocyte factor-1 (Pref-1)(Canalis, 2005). Glucocorticoids also inhibit genes involved in bone formation such as collagen type 1 (Col1a1) and Runx2, or through antagonizing bone morphogenic protein- (BMP-) and wingless/integrated- (Wnt-) signaling pathways (Canalis et al., 2007). Glucocorticoids also inhibit osteoblast function by suppressing the expression of

insulin-like growth factor 1 (IGF-1) which is required for collagen synthesis (Canalis, 2005). These actions are mediated through the activation of GR, a nuclear receptor that alters gene expression by binding as dimers to GC-responsive elements in genes like nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), activator protein 1(AP-1), interferon regulatory transcription factor 3 (IRF-3) or signal transducer and activator of transcription 5 (STAT5) (Kassel & Herrlich, 2007), or through monomeric GR activity (Rauch et al., 2010). Glucocorticoids also increase osteoclastic bone resorption by increasing RANKL expression and reducing OPG expression by osteoblasts. Enhanced bone resorption is only transient; prolonged exposure lowers osteoclast numbers and resorption (Canalis & Delany, 2002; Henneicke et al., 2014; Mitra, 2011). These data suggest that glucocorticoids induce BMD decrease primarily by acting on osteoblasts. The glucocorticoid dexamethasone down-regulates osteocalcin in bone cells (S.-M. Chen et al., 2018) and in osteogenic media strongly induces adipogenesis in bone marrow stromal cells, in part via induction of PPARγ (Ghali et al., 2015). In fact GR and PPARγ cross-talk is largely synergistic, particularly in the case of lipogenesis where GR is acting via PPARγ (Hasan et al., 2018).

PPARγ is the primary transcription factor regulating adipogenesis and *in vivo* exposures to PPARγ agonists have been shown to induce adipogenesis and suppress osteogenesis in mouse BM-derived MSCs (Watt & Schlezinger, 2015). Increased bone marrow adiposity is associated with lower bone strength and

quality (Bredella et al., 2011; Moerman et al., 2004). PPARγ is part of the nuclear receptor superfamily that includes steroid, retinoid an thyroid hormone receptors, and is most highly expressed in adipose tissue (Mangelsdorf et al., 1995). In addition to regulating adipocyte differentiation, PPARγ plays key roles in lipid storage and mobilization, glucose metabolism and inflammatory responses (Janani & Ranjitha Kumari, 2015). PPARγ also plays a crucial role in regulating MSC differentiation. PPARγ not only induces the expression of adipogenic genes, but osteoblast differentiation also requires the inhibition of PPARγ as PPARγ inhibits the expression of genes required for osteogenic differentiation (Wan, 2010). There is evidence that exposure of MSCs to chemicals that act as PPARγ agonists can promote adipogenesis and reduce bone formation (Watt & Schlezinger, 2015). Importantly studies have shown that compounds in OSPW or other oil-extraction wastewater can induce PPARγ activity and subsequently increase adipogenesis (Kassotis et al., 2018; Peng et al., 2016). However, it remains to be elucidated whether the induction of PPARγ activity by compounds in OSPW can have an effect on the bone strength through the differentiation of progenitor cells into osteoblasts.

1.2. Main Objective

The differentiation of progenitor cells into osteoblasts is central to the ability of bone to perform its primary structural function, adapt to loads, and

repair damage throughout the life-span of mammals. Osteoblasts originate from the same pool of progenitor cells as bone marrow adipose tissue, and the differentiation into mature osteoblasts is influenced by many endocrine factors including glucocorticoids and sex hormones. Consequently, NAFCs of OSPW, which contain endocrine disrupting chemicals, may be pushing progenitor cells away from osteoblastic differentiation and toward adipogenesis. This may then contribute to the lower bone strength observed in free-living river otters trapped in the AOSR. Therefore, the overall goal of this thesis was to test the hypothesis that exposure to NAFC can alter the differentiation of MSC progenitor cells to favour reduced bone formation and bone quality. Specifically, this thesis examined the effects of NAFCs on osteoblast differentiation and adipocyte differentiation and sought to identify mechanisms by which these effects occur to further our understanding of the impact OSPW related contaminants are having on wildlife in areas affected by mining activity.

2. Chapter 2

Prepared for publication.

2.1. Preface and significance to thesis

Our lab and collaborator Dr. Phillipe Thomas found that there was a decrease in baculum strength associated with proximity to oil sands mining activity in north American river otters (*Lontra canadensis*) (Thomas et al., 2021). However, the contribution of NAFCs to this effect and the mechanism by which PRCs were impairing bone strength was unknown. We had also received the naphthenic acid fraction of OSPW collected from a tailings pond from Dr. Richard Frank and Dr. Mark Hewitt from Environment and Climate Change Canada. Thomas et al. 2021 were not able to measure NAFC exposure in their study, but as they are considered to have significant toxicity, we examined the effects of NAFCs on osteoblast development and function. This work demonstrates that NAFC can inhibit osteoblast differentiation, which is required for bone formation, and acts at least in part via GR activity. Interestingly the inhibition of osteoblast differentiation was greater with NAFC treatment than with the GR activator positive control dexamethasone. This work demonstrates that exposure to NAFCs may impair bone strength and this effect should be investigated in *in vivo* models.

2.2. Author contribution

RMG, PJT and ACH were responsible for the experimental design of this study. LMH and RAF provided the NAFC stock solution. RMG was the primary contributor to the data in Figure 1, Figure 2, Figure 3, and Figure 4. LJ helped with preliminary lab experiments. RMG wrote the first draft of the manuscript and the rest of the authors provided edits and feedback on the manuscript.

*2.3. **Naphthenic acid fraction components from oil sands process affected water from the Athabasca Oil Sands Region impair murine osteoblast differentiation and function***

Naphthenic acid fraction components from oil sands process affected water from the Athabasca Oil Sands Region impair murine osteoblast differentiation and function

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Short title: Naphthenic Acid Fraction Components Inhibit Osteoblastogenesis

Key words: Naphthenic acids, oil sands process-affected water, osteoblast, osteoblastogenesis, osteoblast differentiation, glucocorticoid receptor

Abstract

The extraction of bitumen from surface mining in the Athabasca Oil Sands Region (AOSR) produces large quantities of Oil Sands Process-affected Water (OSPW) that need to be stored in settling basins near the extraction sites. Compounds in OSPW impair bone health in some organisms, which can lead to increased fracture risk and lower reproductive fitness in wildlife. Naphthenic acid fraction components (NAFCs) are among the most toxic class of compounds in OSPW, however the effect of NAFCs on osteoblast development is largely unknown. In this study we demonstrate that NAFCs from OSPW inhibit osteoblast differentiation and deposition of extracellular matrix which is required for bone formation. Extracellular matrix deposition was inhibited in osteoblasts exposed to 12.5-125 mg/L of NAFC for 21 days, concentrations that had no effect on cell viability. We also show that components within NAFCs inhibit the expression of gene markers of osteoblast differentiation and function, namely alkaline phosphatase, osteocalcin and collagen type 1 alpha 1 (*Col1a1*). These effects were partially mediated by the induction of glucocorticoid receptor activity; NAFCs induce the expression of the GR activity marker genes *Sgk1* and *p85a,* and inhibits GR protein and *Opg* RNA expression. This study provides evidence that NAFC concentrations ≥12.5mg/L can directly act on osteoblasts to inhibit bone formation and suggests that NAFCs contain components that can act as GR agonists which may have further endocrine disrupting effects on exposed wildlife.

Introduction

The Athabasca Oil Sands Region (AOSR) is one of the world's largest deposits of extractable bitumen (Alberta Energy Regulator, 2015). The process of bitumen extraction uses large amounts of water that becomes OSPW; this OSPW is then stored in tailings ponds and cannot be discharged into receiving waters after use (Wu et al., 2019). There is concern that chemicals from bitumen extraction may be affecting development and reproductive health of animals in areas impacted by mining activity; in particular many of these concerns are centred around the toxicity of oil sands process affected water (OSPW). However, there is concern that components of OSPW can migrate from tailings ponds into nearby wetlands through groundwater mixing or seepage from tailings ponds.

OSPW contains a variety of compounds including naphthenic acid fraction components (NAFCs) which are a large and diverse group of organic acids that are natural components of bitumen (Clemente & Fedorak, 2005). NAFCs are considered the most toxic class of organic compound in OSPW (C. Li et al., 2017). As a result, the toxicity of NAFCs has been widely studied. Indeed, there is a considerable amount of evidence demonstrating the toxicity of OSPW on

invertebrates (Bartlett et al., 2017) and fish (Mahaffey & Dubé, 2016; Marentette et al., 2015, 2017). Although few studies have investigated the effects that chronic exposure to sub-toxic concentrations of NAFCs have on major organ systems in mammals (Clemente & Fedorak, 2005; C. Li et al., 2017), there is considerable evidence that exposure to OSPW and NAFC can act as endocrine disrupting compounds. Sub-toxic effects of NAFCs that have been observed include alterations in sex hormone and glucocorticoid production (Knag et al., 2013; Lister et al., 2008), impaired development and reproductive success and reduced secondary sexual characteristics in animals exposed to NAFC (Kavanagh et al., 2013; C. Li et al., 2019; Philibert et al., 2019; Rogers et al., 2002).

More recently, in a study investigating the baculum strength of animals in the AOSR, lower baculum strength was observed in river otters (*Lontra canadensis*) collected in areas with high levels of mining activity compared to those collected in areas far from mining activity (Thomas et al., 2021). A decrease in baculum strength can reduce the reproductive fitness of male mammals, thereby presenting a risk to the stability of these wild populations (Brassey et al., 2018; Sonne et al., 2015; Stockley et al., 2013). Previous studies have also shown that exposure to endocrine disrupting chemicals lower baculum bone mineral density (BMD) (Sonne et al., 2015). Although, there is limited information regarding the effects of OSPW or NAFC on mammalian bone, a study in fathead minnow larvae reported that both a commercial technical naphthenic acid mixture and NAFC of tailings water from the AOSR induced skeletal deformities and

altered gene networks related to chondrocyte development and endochondral ossification (Loughery et al., 2019). These data suggest that it is biologically plausible that NAFC may also impact mammalian bone development.

Bone strength is maintained throughout life by the process of remodeling, which allows bone to adapt and repair microdamage through the resorption of old bone and deposition of new bone matrix (Seeman, 2003). This process is regulated by endocrine factors and paracrine signaling between osteocytes, osteoblasts and osteoclasts (Hauge et al., 2001). Osteoblasts synthesize new bone matrix on bone-forming surfaces as they differentiate from osteoblast progenitor cells during the formation phase of the remodelling cycle (Komori, 2016). The primary endocrine pathways regulating BMD are parathyroid hormone (PTH) (Silva & Bilezikian, 2015; Stein et al., 2013), insulin-like growth factor 1 (IGF-1)(McClung et al., 2014; Plotkin & Bellido, 2016) and glucocorticoids (Rauch et al., 2010; Weinstein et al., 1998). Glucocorticoid receptor (GR) activation in osteoblasts inhibits differentiation and production of extracellular matrix (ECM) (Rauch et al., 2010). Further, the use of synthetic glucocorticoids like dexamethasone is also the most common form of secondary osteoporosis (Briot & Roux, 2015). Exposure to OSPW has been found to increase serum cortisol levels (Lister et al., 2008), but no studies have investigated whether chemicals in OSPW act as agonists for PTH, IGF1 or glucocorticoid receptors. The goals of this study were to 1) investigate the effects of the NAFC extracted from OSPW from an active mining operation within the AOSR (Frank et al., 2006) on the osteogenic

potential of mouse pre-osteoblastic (MC3T3-E1) cells and 2) determine if the underlying mechanism is glucocorticoid receptor dependent.

Materials & methods

NAFC preparation

Naphthenic acid extracts were isolated from tailings pond water collected from Syncrude Canada Ltd. West In-pit settling basin in Fort McMurray, Alberta, Canada in 2009, using a procedure described in (Frank et al., 2006). An NAFC stock solution was prepared and stored in glass vessels in darkness and preserved in 0.05 M NaOH with a final concentration of 2504 mg/L determined via liquid chromatography/quadrupole mass spectrometry with time-of-flight detection (LC/QTofF) as previously described in Marentette et al., (2017).

MC 3T3-E1 cell culture & treatment

MC 3T3-E1 subclone 4 mouse newborn fibroblasts (ATCC, Manassas, VA, USA) were maintained in ascorbic acid-free alpha minimum essential medium (aMEM) with ribonucleosides, deoxyribonucleosides, 2mM L-glutamine and 1 mM sodium pyruvate (Gibco, Waltham, MA, USA), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% P/S. At 70% confluence cells were passaged to 6-well plates and left to reach confluence (100%). 48hrs after reaching 100% confluence (day 0), differentiation was induced with 100μM L-ascorbic acid (EMD, Mississauga, ON, Canada) and 2mM

β-glycerophosphate (Sigma, St. Louis, MO, USA) (Wang et al., 1999) added to the supplemented aMEM media (differentiation media). To investigate whether NAFC inhibits osteoblastogenesis, cells were treated beginning on day 0 with 125 mg/L, 25 mg/L, 12.5 mg/L and 1.25 mg/L of the NAFC mixture that was diluted in differentiation media (described above). These concentrations encompass the range of NAFCs that have been reported in surface waters, wetlands, and tailings ponds in the AOSR (Vander Meulen et al., 2021). Dexamethasone (1µM, Sigma), a glucocorticoid receptor agonist, was used as a positive control to assess the effects of GR activation during differentiation (Rauch et al., 2010). The pH of the media was adjusted to be similar across all groups using NaOH or HCl after the addition of NAFC. Cells were treated for 7, 14, or 21 days after the initiation of differentiation; media was changed every 4 days.

Cell viability

Cells were seeded and treated in 96-well plates following the protocols described above. After 7 and 14 days of treatment, 10% alamarBlue reagent (BioRad Laboratories, Hercules, CA, USA) was added to fresh media and cells were incubated for 2h. Absorbance was then read at 570nm in a plate reader (Synergy H1 microplate reader, Norgen BioTek, Thorold, ON, Canada) with a reference read at 600nm. Treatments were considered cytotoxic if the average absorbance for the group was below 80% of the control group.

ALP staining and enzyme activity assay

Alkaline phosphatase (ALP) is an early marker of osteoblast differentiation (Lee et al., 2017). To visualize ALP abundance, after 14 days of treatment cells were washed with PBS and fixed in 10% formalin for 10 min. The fixed cells were incubated in a solution containing 1mg/ml Fast Blue RR salt and 0.4 mg/mL naphthol AS-MX phosphate disodium salt (Thermo Fisher Scientific, Waltham, MA, USA) at pH 8.4 for 15 min. The reactions were stopped by rinsing with deionized water and cells were photographed under a light microscope (Motic Microscopes, Xiamen, China). To measure ALP activity, an enzymatic ALP activity assay (Abcam, Cambridge, UK) was used according to the instructions provided by the manufacturer. Briefly, cells were harvested with a cell lysis solution, a p-nitrophenyl phosphate (pNPP) phosphatase substrate was applied to the samples, and absorbance was read at 405nm using a microplate reader (Norgen BioTek). Absorbance data was corrected to background and compared to control.

Alizarin Red S staining of MC3T3-E1 osteoblasts

To measure calcium deposition, after 21 days of treatment, cells were washed with Dulbecco's phosphate-buffered saline (DPBS), then fixed with 10% formalin and stained with 2% Alizarin Red S (ARS) solution (EMD) for 15 min. The ARS solution was removed, and the cells were then washed 5x with water. Representative images were taken using phase-contrast microscopy (Motic

Microscopes). The dye was extracted using 10% acetic acid, cells were separated, and supernatant was transferred to a 96 well plate and absorbance was read at 405nm in a microplate reader (Norgen BioTek). Absorbance data was corrected for background and compared to control.

Real-time quantitative PCR

After 7, 14 or 21 days of treatment, cells were washed with phosphate-buffered saline (PBS) and RNA was extracted using TRIzol reagent (Invitrogen, Waltham, MA, USA). RNA concentrations were determined using the NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific). Complementary DNA (cDNA) was made from 2 μg of mRNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham MA, USA) as per the manufacturer's instructions. Real-time quantitative PCR (RT-qPCR) was carried out using PerfeCTa SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD, USA) on the CFX384 Touch Real Time PCR Detection System (Bio-Rad Laboratories). The PCR cycling settings included polymerase activation (95 °C for 10 m), followed by 40 cycles of denaturing (95 °C for 15s) and annealing/elongation (60 °C for 1m). Levels of gene expression were generated using the 2(-ΔΔC(T)) method (Livak & Schmittgen, 2001) and normalized using the geometric means of two reference genes: Tyrosine 3-monooxygenase/tryptophan 5-monooxygnase activation protein zeta *(Ywhaz)* and peptidylprolyl isomerase A (*Ppia)*. Gene targets for osteoblast differentiation:

alkaline phosphatase *(Alp)*, collagen type 1 alpha 1 chain *(Col1a1)*, *Osteocalcin*, and runt-related transcription factor 2 *(Runx2)*, glucocorticoid receptor activity: serum/glucocorticoid regulated kinase 1 *(Sgk1)*, receptor activator of nuclear factor kappa-B ligand (*Rankl)*, osteoprotegerin (*Opg)* and phosphatidylinositol 3-kinase regulatory subunit alpha (*p85a)* were measured. Primer sequences are in Table 1.

Immunoblotting

After 21 days of treatment, cells were isolated in lysis buffer (50 mM HEPES, 150 mM NaCl, 100 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol and 1 mM sodium orthovanadate, with 1% Triton X and one tablet of cOmplete Protease Inhibitor Cocktail (Roche, Basil, Switzerland) per 50 ml) then stored at −80 °C until analysis. Protein concentration was determined with the Pierce BCA Protein Assay kit (Thermo Fisher Scientific). Lysates were then diluted with Laemmli buffer and run on a 10% polyacrylamide gel (Bio-Rad Laboratories) to separate proteins based on size. Samples were then transferred to a polyvinylidene difluoride (PVDF) membrane and blocked in 5% BSA for 1h at room temperature (20–25 °C). Membranes were incubated with primary antibody (1:1000) overnight at 4 °C. Appropriate secondary antibodies were used at a concentration of 1:10000. Bound antibodies were detected using Clarity Western ECL Substrate (BioRad Laboratories). For primary antibody information see Table 2.

Statistical analysis

Data are expressed as the means +/- standard errors of the means (SEM). Statistical analyses were carried out using GraphPad Prism 9.2 (GraphPad Software Inc, San Diego, CA, USA). One-way analysis of variance (ANOVA) was followed by post-hoc testing (Dunnett multiple comparisons) when significance was indicated ($p<0.05$).

Results

NAFC inhibits osteoblastogenesis in murine pre-osteoblasts

Treatment with all doses of NAFC for 8 and 14 days had no impact on the viability of MC3T3-E1 pre-osteoblast cells (Fig. 1). Following 14 days of treatment with NAFC, ALP activity was assessed by ALP activity assay and staining. Treatment with 125mg/L NAFC as well as 1μM dexamethasone led to significantly lower ALP activity than in control cells (Fig. 2a-b). The deposition of calcium in a mineralized ECM is a common functional marker for the late stages of osteogenic differentiation. We measured the deposition of calcium in 3T3-E1 cells following 21 days of treatment with NAFC using Alizarin Red-S (ARS) calcium staining. Treatment with 12.5-125 mg/L of NAFC resulted in a dose-dependent reduction in extracellular calcium deposition (Fig. 2c-d).

To further evaluate the effect that NAFC has on osteoblastogenesis, the expression of markers of osteoblast differentiation *Runx2, Alp, osteocalcin* and *Col1a1* were measured after 7-, 14- and 21-days of exposure. NAFC had no impact on *Runx2* gene or protein expression at any time point (Fig. 3a, f), but the 12.5-125mg/L doses of NAFC induced significant reductions in the expression of *Alp, osteocalcin* and *Col1a1* genes at the 14- and 21-day time points relative to control treated cells (Fig. 3b-d). The lowest dose of NAFC significantly inhibited *Alp* gene expression at 7 days only. At 14 and 21 days, NAFC exposures resulted in significantly lower Alp expression at 12.5, 25 and 125mg/L doses (Fig. 3b). Similarly, 125mg/L of NAFC significantly inhibited *osteocalcin* expression at all

time points, and by 21 days all doses of NAFC had significantly lower *osteocalcin* expression (Fig. 3c). Accordingly, we observed significantly lower osteocalcin protein expression in cells exposed to 125mg/L of NAFC than control cells (Fig. 3e). *Col1a1* expression was unchanged after 7 days of NAFC treatment, but after 14 days cells treated with 12.5mg/L of NAFC and higher exhibited significantly lower Col1a1 expression than control cells, which then persisted to 21 days in cells treated with 125mg/L of NAFC (Fig. 3d).

NAFC inhibition of osteoblastogenesis is mediated by glucocorticoid receptor activity

Given that GR activity has a common and strong inhibitory effect on osteoblastogenesis, we sought to determine if GR was implicated in the mechanism by which NAFC inhibits osteoblastogenesis. We measured the gene expression of downstream markers of GR activity *Opg*, *Rankl* after 14 days of NAFC treatment and *Sgk1* and *p85a* after 14 and 21 days of treatment. We observed a dose-depended decrease in *Opg* expression as well as a significant increase in *Rankl* expression in the 125mg/L NAFC treated cells, resulting in a significant increase in the *Rankl*/*Opg* ratio at 14 days (Fig. 4a-c). We also observed significant increases in *Sgk1* gene expression after both 14 and 21 days of treatment with 125mg/L of NAFC, and after 14 days of treatment with 1.25mg/L of NAFC (Fig. 4d-e). *P85a* expression was significantly higher with 14 days of NAFC treatment in a dose-dependent manner, an effect which persisted at

21 days (Fig. 4f-g). We then measured protein markers of GR activity after 21 days of treatment with NAFCs and found that there was no change in OPG or RANKL protein expression with NAFC or the 1μM dexamethasone GR agonist positive control, but there was a significant decrease in GR protein expression in cells treated with both 125mg/L of NAFC and dexamethasone (Fig. 4h-l). These results are consistent with an induction of GR activity by NAFC components functioning as GR agonists.

Discussion

Although there are numerous studies that have investigated the toxicological effects of environmental contaminants derived from oil and gas production (C. Li et al., 2017; Rodríguez-Estival & Smits, 2016), few of these have focused on bone health. Two studies have found skeletal deformities caused by OSPW or light oil fraction on bone in fish (Danion et al., 2011; Loughery et al., 2019) and another found that river otters in parts of the AOSR where mining activity is prevalent have weaker baculums than otters farther from mining activity (Thomas et al., 2021). In the present study we found that treatment with NAFCs derived from the AOSR are sufficient to inhibit osteoblast differentiation, determined by measuring calcified ECM, ALP activity and gene and protein markers of osteoblastogenesis. We also found NAFC exposure results in altered expression of components of GR signalling similar to what is seen with a known GR agonist dexamethasone.

In the current study, we found that treatment with NAFC inhibited the differentiation of precursor cells into mature osteoblasts. Treatment with 12.5-125mg/L of NAFC significantly decreased the deposition of extracellular matrix in differentiating osteoblasts (Fig. 2d), indicating an inhibition in the differentiation and primary function of these cells. These findings are in line with previous work in which exposure to the water-soluble fraction of light cycle oil, which contains several petroleum related compounds, resulted in lower bone mineralization in sea bass (Danion et al., 2011). Further, research from other

groups has demonstrated that commercial NA mixtures and NA fraction components of OSPW increase spinal deformities and skeletal gene networks of chondrocyte development and endochondral ossification, osteoclast activation and function in fathead minnow larvae (Loughery et al., 2019). Similarly, the data presented here demonstrates that NAFCs have a direct effect on osteoblasts.

In this study we also show that NAFC can activate GR in a manner similar to dexamethasone. Glucocorticoids like dexamethasone inhibit osteoblast mineral deposition and ALP activity through inducing GR receptor activity (Rauch et al., 2010). NAFC and dexamethasone treatment resulted in similar changes in the expression of GR protein, *Opg* and *p85a* RNA (Fig. 4a,f), suggesting NAFC is also acting as a GR agonist (Kuo et al., 2012; Xu et al., 2019). However, compounds in the NAFC mixture were less potently inducing *Sgk1* expression to a lower degree than to dexamethasone ($p=0.0119$) (Fig.4e). This is despite the fact that the inhibition of ECM deposition was more profound in cells treated with NAFC vs dexamethasone (92.7% lower ECM deposition with 125mg/L NAFC treatment than CTL vs 25.3% lower ECM deposition with dexamethasone treatment than CTL, $p<0.0001$) (Fig. 2d). Previous studies that have investigated the effects of NAFCs on gene markers of skeletal development did not measure the expression of the markers of osteoblastogenesis *Alp*, *Col1a1* or *osteocalcin* (Loughery et al., 2019). The lower expression of *Alp*, *Col1a1* and *osteocalcin* that we observed (Fig. 3b-d) are consistent with what others have reported in osteoblasts or mesenchymal stem cells treated with glucocorticoids (Chen et al.,

2018; H. Li et al., 2015). Due to the fact that the NAFC is a mixture of several thousand organic acids, various components of the NAFC mixture could be functioning through multiple pathways that contribute to the totality of responses observed with the whole mixture. It is likely that some components of the NAFCs may be inhibiting osteoblast differentiation through other mechanisms in addition to the those acting via the GR pathway. NAFC from other sources have previously been shown to alter other pathways that can affect BMD, including altered sex hormone function (Knag et al., 2013; Lister et al., 2008) and increased inflammatory cytokine expression (Garcia-Garcia et al., 2011); the effects of our NAFC mixture on these pathways remains to be determined. Our data suggest that NAFC is acting through GR, but NAFC is a complex mixture and we have not ruled out whether it is also acting through PTH, wingless-related integration site (Wnt) or peroxisome proliferator-activated receptor gamma (PPARγ), or other pathways that can inhibit osteoblastogenesis.

Although our findings are consistent with the hypothesis that NAFC exposure is inhibiting osteoblast differentiation and function, our observations were in a cell line and thus further study examining the effect of NAFC exposure on bone *in vivo* are required. A major limitation of studies investigating the toxicological effects of NAFC is the challenge of measuring the tissue concentrations and compositions of NAFC in wildlife. However, the concentrations of NAFCs used in this study are similar to those used in other toxicological studies (Bartlett et al., 2017) and encompass the range of

concentrations reported in surface water, industrially-affected wetlands and tailings ponds in the AOSR (Vander Meulen et al., 2021). Finally, while we found evidence of GR activity being induced by NAFC, we have not confirmed that this is the only mechanism of action.

In summary, we have shown that exposure to NAFCs has the potential to inhibit bone formation via inhibition of osteoblast differentiation. If our findings are confirmed *in vivo*, NAFC derived from OSPW may reduce bone strength of chronically exposed mammals which could compromise fitness and reproductive health.

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Declaration of Competing Interest

None.

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Disclaimer

The views in this paper are only held by the authors and are not representative of the official policy of the authors' individual affiliations.

Figures

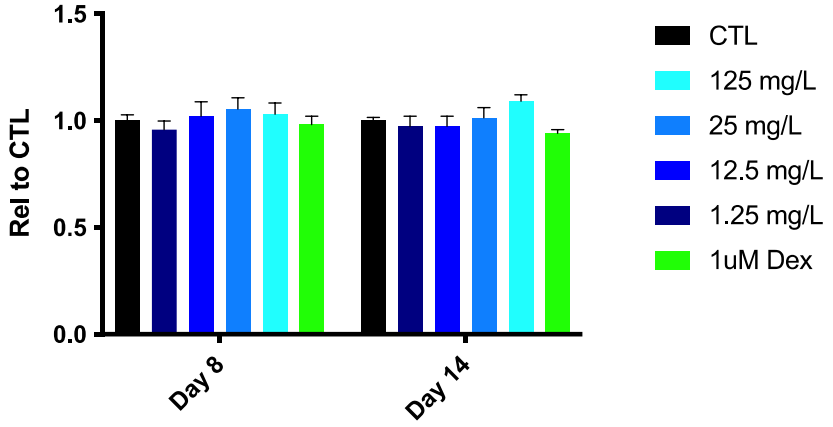

Figure 1: Effects of NAFC on cell viability.
Cell viability of MC3T3-E1 osteoblastic cells after 8 and 14 days of treatment with of concentrations of NAFC from 1.25 mg/L to 125 mg/L and 1μM dexamethasone positive control.
Each value is the mean +/- SEM (n=6). *$p < 0.05$, compared with the corresponding control group at each time point.

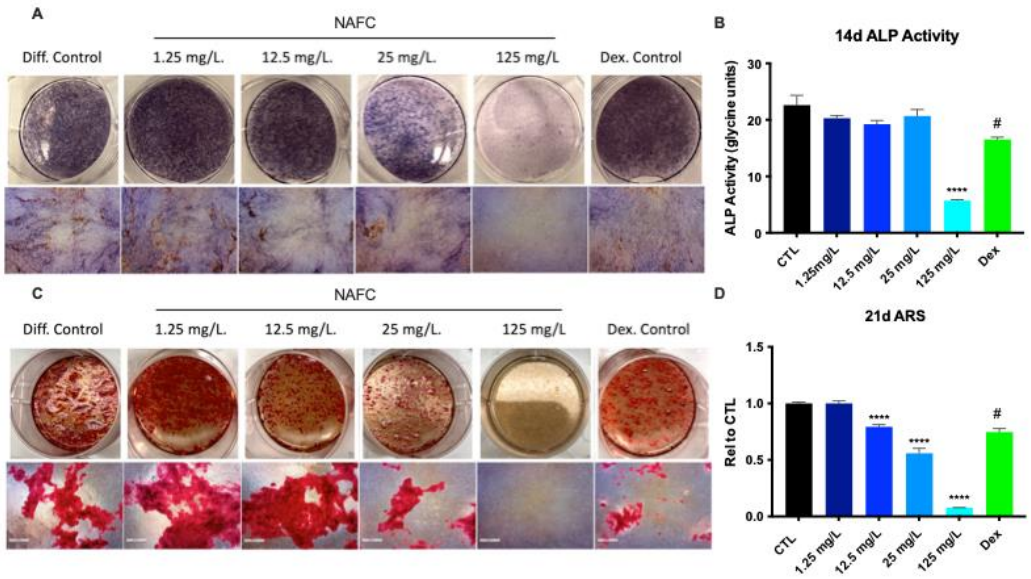

Figure 2. NAFC inhibits osteoblast differentiation in MC3T3-E1 cells in a dose dependent manner.

(A) ALP staining representative images in MC 3T3-E1 cells treated for 14 days with 1.25-125mg/L NAFC and 1μM dexamethasone positive control.

(B) ALP activity of MC 3T3-E1 cells treated for 14 days with 1.25-125mg/L NAFC and 1μM dexamethasone positive control.

(C-D) ARS calcium staining assay results and representative images in MC 3T3-E1 cells treated for 21 days with 1.25-125mg/L NAFC and 1μM dexamethasone positive control.

Each value is the mean +/- SEM (n=6). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, #$p < 0.05$ positive control, the NAFC treated group compared with the differentiation control group.

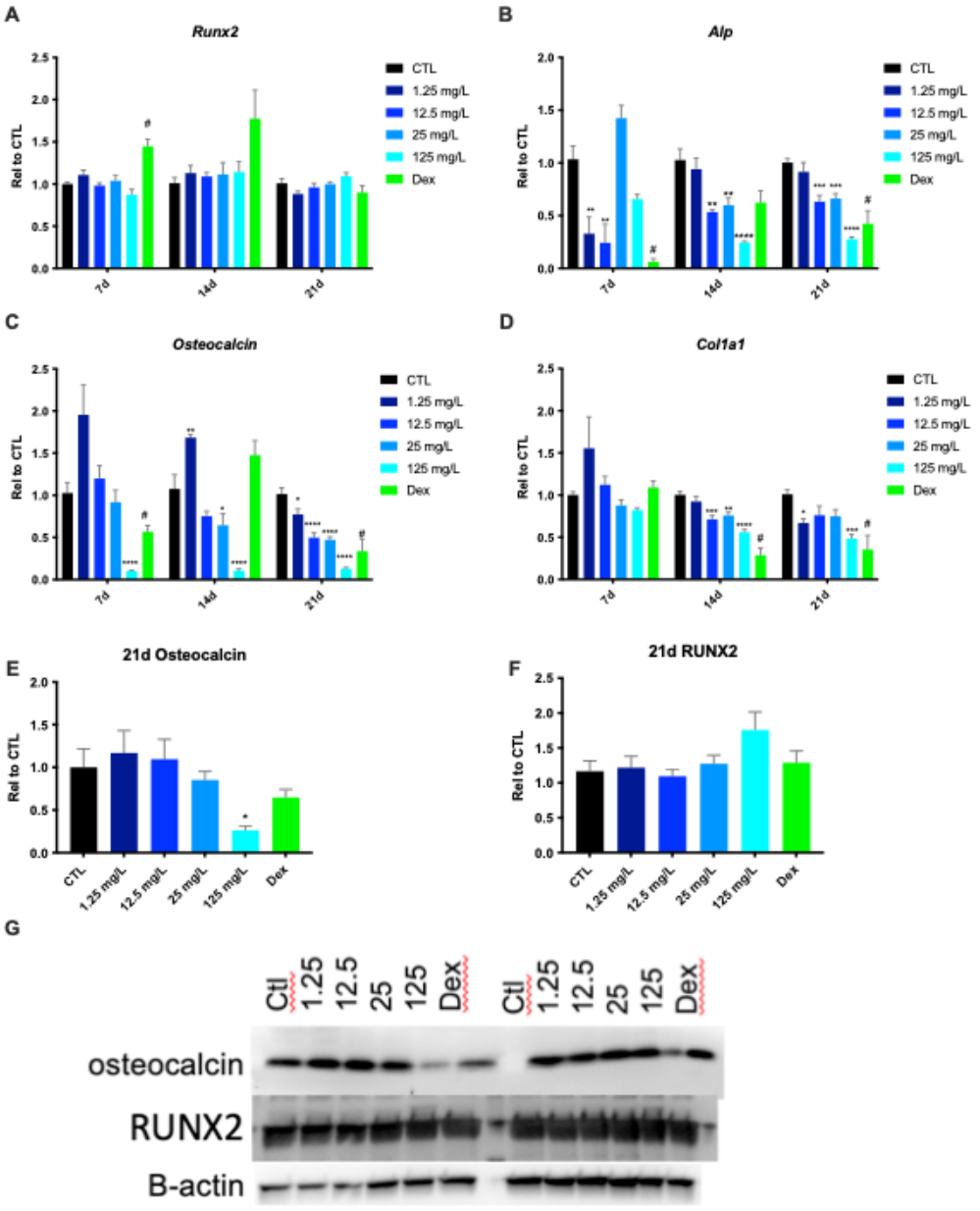

Figure 3. Effects of NAFC on osteoblast markers during differentiation of MC3T3-E1 cells.

(A-D) Expression of osteoblastic marker genes *Runx2*, *Alp*, *osteocalcin* & *Col1a1* in MC 3T3-E1 cells treated for 7, 14 and 21 days with 1.25-125mg/L NAFC and 1μM dexamethasone positive control.

(E-G) Protein concentration and western blot representative images of osteocalcin and RUNX2 in MC 3T3-E1 cells treated for 21 days with 1.25-125mg/L NAFC and 1μM dexamethasone positive control.

Each value is the mean +/- SEM (n=6). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, #$p < 0.05$ positive control, the NAFC treated group compared with the differentiation control group.

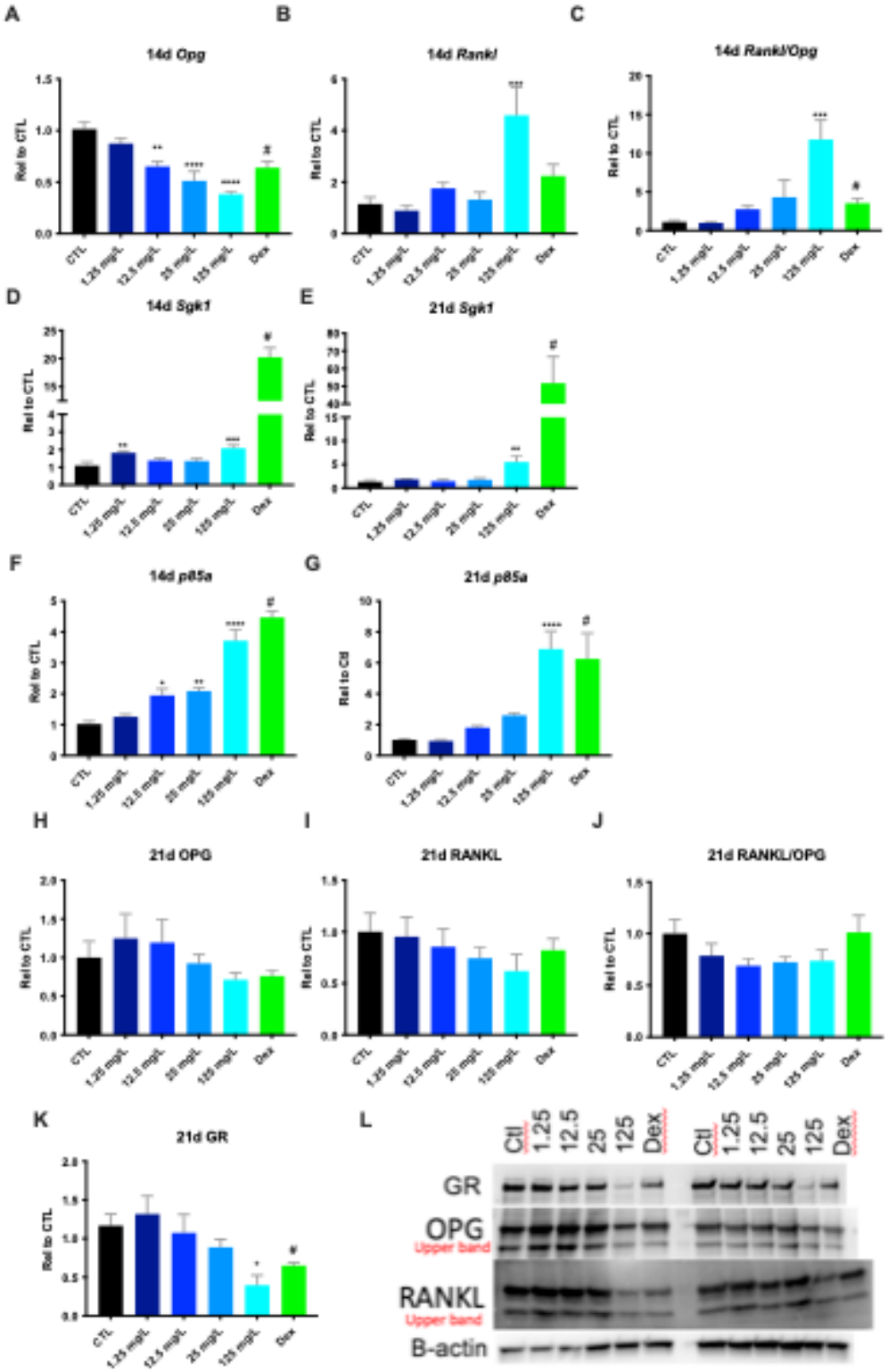

Figure 4. NAFC induce glucocorticoid receptor activity in MC3T3-E1 cells.

Expression of glucocorticoid receptor acivity markers in MC3T3-E1 cells treated with 1.25-125mg/L NAFC for 14 and 21 days with 1μM dexamethasone positive control.
(A-C) RNA levels of *Opg* and *Rankl*, and *Rankl/Opg* ratio after 14 days of NAFC treatment.
(D-G) RNA levels of *Sgk1* and *p85a* after 14 and 21 days of NAFC treatment.
(H-J) Protein levels of OPG, RANKL and RANKL/OPG ratio following 21 days of NAFC treatment.
(I) Protein levels of GR protein following 21 days of NAFC treatment.
(J) Western blotting representative images.
(K-L) RNA levels of *Lgr5* after 14 and 21 days of NAFC treatment.
Each value is the mean +/- SEM (n=6). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, #$p < 0.05$ positive control, the NAFC treated group compared with the differentiation control group.

Table 1: Table of primers.

Gene	Forward/ Reverse	Sequence	Accession No.
ALP	Forward	5'-AGCAGGTTTCTCTCTTGGGC-3'	NM_007431.3
ALP	Reverse	5'-GGTGCTTTGGGAATCTGTGC-3'	
COL1A1	Forward	5'-GGTGGGGTGGGAAGGAATTT-3'	NM_007742.4
COL1A1	Reverse	5'-GGTCTAGGGAGCATCTCAGC-3'	
IGF1	Forward	5'-CAGGAGGGTGCAACATCAGA-3'	NM_010512.5
IGF1	Reverse	5'-GTGGCATCCCAGTGACAGAT-3'	
LGR5	Forward	5'-CAAGCCCTGTGAGCACCTAT-3'	NM_010195.2
LGR5	Reverse	5'-TCTGAACACGGTCAAAGCCA-3'	
OPG	Forward	5'-GGAACCCCAGAGCGAAACAC-3'	NM_008764.3
OPG	Reverse	5'-GCCAAATGTGCTGCAGTTCG-3'	
Osteocalcin	Forward	5'-TTCTGCTCACTCTGCTGACC-3'	NM_007541.3
Osteocalcin	Reverse	5'-TATTGCCCTCCTGCTTGGAC-3'	
p85a	Forward	5'-CAAAGCGGAGAACCTATTGC-3'	NM_001024955.2
p85a	Reverse	5'-ATAGCAGCCCTGCTTACTGC-3'	
PPIA	Forward	5'-GACAAAGTTCCAAAGACAGCAGAA-'3	NM_008907.2
PPIA	Reverse	5'-CCAAATCCTTTCTCTCCAGTGC-3'	
RANKL	Forward	5'-AGGGAGCACGAAAAACTGGT-3'	NM_011613.3
RANKL	Reverse	5'-GGAAGGGTTGGACACCTGAA-3'	
RUNX2	Forward	5'-GCCTCCAGCACCCTATACCC-3'	NM_001271631.1
RUNX2	Reverse	5'-CACATAGGTCCCCATCTGCC-3'	

SGK1	Forward	5′-GGGTGCCAAGGATGACTTTA-3′	NM_001161845.2
SGK1	Reverse	5′-CTCGGTAAACTCGGGATCAA-3′	
YwHAZ	Forward	5'-GGGGTGATTGGCAAAAGGTA-3'	NM_001356569.1
YwHAZ	Reverse	5'-CGACTTGGAAGCACAGAACT-3'	

Table 2: Table of antibodies.

Antibody	Manufacturer	Product Number
β-actin	Cell Signaling Technology	4967
GR	Santa Cruz Biotechnology	sc-1004
Osteocalcin	abcam	ab93876
RANKL	R&D Systems	AF462
RUNX2	abcam	ab76956

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3. Chapter 3

Prepared for publication.

3.1. Preface and significance to thesis

Our lab had previously found that commercial naphthenic acids (NAs) were PPARγ ligands (*Jamshed and Holloway, unpublished data*), and the induction of PPARγ activity by environmental contaminants has previously been demonstrated to induce adipogenesis in adipocyte progenitor cells (Boucher et al., 2014). In addition, OSPW (which contains naphthenic acids) had previously been found to contain PPARγ ligands and induce adipogenesis in adipocyte progenitor cells (Peng et al., 2016), Dr. Richard Frank and Dr. Mark Hewitt at Environment and Climate Change Canada generously donated a sample of NAFC of OSPW from oil sands tailings water in the AOSR which had not been assessed for its effects on adipogenesis. We found that although commercial NAs were PPARγ ligands, they did not induce adipogenesis, nor did NAFC. Interestingly, we found that the highest dose of NAFC inhibited the expression of gene markers of PPARγ activation *Fabp4, Cd36* and *C/ebp-β* in mature adipocytes, suggesting a potential inhibition of PPARγ by NAFC.

3.2. Author Contribution

RMG, PJT, LJ and ACH were responsible for the experimental design of this study. LMH and RAF provided the NAFC stock solution. RMG was the primary contributor to the data in Figure 2, Figure 3, Figure 4, and Figure 4. LJ

was the primary contributor to the data in Figure 1 and she and Raji Rajilingham contributed to preliminary lab experiments. RMG wrote the first draft of the manuscript and the rest of the authors provided edits and feedback on the manuscript.

3.3. ***Naphthenic acid fraction components from oil sands process affected water from Athabasca Oil Sands Region do not induce adipogenesis in 3T3-L1 cells***

Naphthenic acid fraction components from oil sands process affected water from Athabasca Oil Sands Region do not induce adipogenesis in 3T3-L1 cells

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Short title: Napthenic Acid Fraction Componenents Do Not Induce Adipogenesis

Key words: Naphthenic acids, oil sands process affected water, OSPW, adipocyte, adipogenesis, lipid metabolism, PPAR gamma

Abstract

The Athabasca Oil Sands Region (AOSR) contain naturally occurring bitumen which is extracted from sand, clay and water through oil sands mining processes. Bitumen contains several potential toxicants including naphthenic acid fraction components (NAFCs) that are solubilized and concentrated in oil sands process affected water (OSPW) during bitumen extraction processes. OSPW is then stored within the AOSR in tailings ponds. NAFCs are considered among the most toxic components in OSPW. OSPW has been reported to induce adipogenesis in adipocytes an effect that has been reported to be mediated via peroxisome proliferator-activated-receptor γ (PPARγ)-mediated signaling. However, the effects of NAFCs on adipogenesis and lipid homeostasis in mature adipocytes is unknown. Therefore, the aims of this study were to 1) evaluate the effect of NAFCs on adipogenesis and lipid metabolism in 3T3-L1 murine adipocyte progenitor cells and 2) determine the effects of NAFCs on the PPARγ signaling pathway. Exposure to environmentally relevant concentrations of NAFCs (1.25-125mg/L) did not affect the transcriptional regulation of adipogenesis, markers of mature adipocytes or lipid content during differentiation. Mature adipocytes treated with NAFCs had decreased expression of markers of PPARγ signalling but this did not result in any changes in cellular lipid content. These data indicate that NAFCs do not induce adipogenesis but may inhibit PPARγ signaling in mature adipocytes. Our data suggests that NAFCs may impair lipid metabolism in adipocytes in chronically exposed animals. Taken

together these data suggest that NAFC exposure may affect lipid homeostasis via direct effects on adipose tissue.

Introduction

Oil and gas extraction in the Athabasca Oil Sands Region (AOSR) accounts for 21% of Alberta's gross domestic product (Government of Canada, 2021). The extraction of bitumen from the oil sands uses large amounts of water (oil sands process-affected water; OSPW) which is used and then stored within the AOSR in tailings ponds (Wu et al., 2019). Tailings ponds are separated from natural waterways and comprise an area in the AOSR of approximately 176km^2 containing $766x10^6$ m^3 of tailings in 2011 (Small et al., 2015). The class of chemicals in OSPW that are considered to have the most toxic effects are the naphthenic acid fraction components (NAFCs). NAFCs are composed of the carboxylic acids and acid-extractable organic compounds of bitumen (Frank et al., 2006; Headley et al., 2011). The effects of exposure to naphthenic acids (NAs) have been widely studied in a variety of invertebrate and vertebrate species (Bartlett et al., 2017; Clemente & Fedorak, 2005; Marentette et al., 2015). However, many of these studies have used commercially available technical NA mixtures as a surrogate for the NAFCs found in OSPW (Bartlett et al., 2017; Brown & Ulrich, 2015). Importantly, technical NA mixtures are less complex with fewer ring structures and oxygen species than NAFCs from OSPW and may have different toxicities (Marentette et al., 2015).

There is increasing concern that exposure to chemicals from oil and gas extraction may be adversely affecting the health of wildlife and human populations living in close proximity to active extraction sites. There is evidence

that many environmental compounds with endocrine disrupting properties (EDCs) have the capability to induce/promote adipogenesis and thus contribute to the development of obesity (Biemann et al., 2021; Gutgesell et al., 2020; Mallhi et al., 2021). Normal adipose tissue functioning is required to adapt to the seasonal variations in food supply, support milk production, and provide endocrine signaling that contributes to fertility, immune function and inflammatory responses (McNamara & Huber, 2018; Wensveen et al., 2015; Young, 1976; Zwick et al., 2018). White adipose tissue (WAT) adapts to energetic stress by increasing the number of adipocytes (hyperplasia), increasing the size of adipocytes (hypertrophy) or involution. Impaired adipose tissue adaptation is associated with chronic inflammation of WAT, accumulation of extracellular matrix and dysregulation of mitochondrial biogenesis, and altered endocrine function of adipocytes (Chouchani & Kajimura, 2019). These attributes of impaired adipose tissue adaptation are associated with a variety of metabolic disorders (Chouchani & Kajimura, 2019). Healthy adipose tissue remodeling requires a balance of hypertrophy and hyperplasia, requiring regulated differentiation of pre-adipocytes into mature adipocytes. Dysregulation of adipocyte proliferation and differentiation is associated with metabolic disease (Muir et al., 2016). There are two populations of adipocyte progenitor cells within the stromal vascular fraction of adipose tissue, mesenchymal stem cells (MSCs) and preadipocytes (Cawthorn et al., 2012). Adipogenesis requires path selection of MSCs to preadipocytes and then differentiation from preadipocytes to

mature adipocytes. Adipocyte differentiation from preadipocytes requires tightly regulated expression of transcription factors including CCAAT/enhancer-binding protein family (C/EBP), sterol regulatory element binding protein-1 (SREBP-1) and peroxisome proliferator-activator receptor γ (PPARγ) (Arimochi et al., 2016).

Many EDCs that have been shown to impact adipogenesis exert their effects via PPARγ signaling. PPARγ is a nuclear receptor and transcription factor central to regulating adipogenesis (Tontonoz et al., 1994). PPARγ agonists induce the transcriptional activation of adipogenesis by interacting with co-activators and co-repressors like the CCAAT/enhancer-binding proteins (C/EBPs) and Krüppel-like factors (KLF)(Rosen & MacDougald, 2006). In addition to its critical role in differentiating adipocytes, PPARγ is also required for normal functioning of adipocyte metabolism and the maintenance of mature adipocytes (Spiegelman, 1997). In mature adipocytes the activation of PPARγ promotes a net flux of fatty acids from circulation into adipocytes and increased insulin sensitivity in adipocytes (Lehrke & Lazar, 2005). Conversely, the inhibition of PPARγ activity in mature adipocytes resulted in a decrease in cell size and triglyceride content and an increase in lipolysis and an increase in the rate of free fatty acid uptake (Tamori et al., 2002).

Several environmental contaminants including bisphenol A, tributyltin, triphenyl phosphate, and triflumizole have been found to induce adipogenesis by activating PPARγ, (González-Casanova et al., 2020). Previous studies have shown that OSPW contains PPARγ ligands and can induce lipid accumulation in

3T3-L1 pre-adipocytes (Peng et al., 2016). Peng et al. (2016) also reported that while the nonpolar fraction of OSPW that should contain most NAFCs did contain PPARγ ligands. However, treatment with a single concentration of this fraction of OSPW did not induce adipogenesis in 3T3-L1 pre-adipocytes in the presence of factors inducing adipogenesis. In this study we expand on this previous work by examining the effects of an environmentally relevant range of doses of isolated NAFCs from the AOSR (Frank et al., 2006) on both 3T3-L1 pre-adipocytes during differentiation in the absence of other factors inducing adipogenesis, and on lipid metabolism of mature adipocytes.

Materials & methods

Naphthenic Acids

Naphthenic acid extracts (NAFC) were isolated from tailings pond water collected from Syncrude Canada Ltd. West In-pit settling basin in Fort McMurray, Alberta, Canada in 2009, using a procedure described in (Frank et al., 2006). A stock solution was prepared in 0.05 M NaOH with a final concentration of 2504 mg/L determined via liquid chromatography/quadrupole mass spectrometry with time-of-flight detection (LC/QTofF) as previously described in Marentette et al., (2017).

MC 3T3-L1 cell culture & treatment

3T3-L1 preadipocytes for adipogenesis assay (AD)

MC 3T3-L1 mouse embryonic fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 1g/L glucose (Corning, Corning, NY, USA), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 1% L-glutamine (Gibco, Waltham, MA, USA), and 1% penicillin/streptomycin (P/S) (Gibco). At 70% confluence, cells were passaged to 6-well plates and left to reach confluence. We exposed 3T3-L1 preadipocytes to NAFCs in the absence of insulin, IBMX and dexamethasone; these factors are known to cause differentiation of 3T3-L1 cells from preadipocytes into mature adipocytes (Zebisch et al., 2012). Two days after 100% confluence (day 0), 3T3-L1 preadipocytes were treated with vehicle (control), 1.25-125mg/L NAFC,

rosiglitazone (2µM, Sigma), and differentiation media. The dose range covers the concentrations of NAFCs that have been reported in surface waters, wetlands and tailings ponds in the AOSR and is in line with other studies investigating the effects of NAFCs have used (Bartlett et al., 2017; Vander Meulen et al., 2021). Two positive control groups were used to compare NAFC treated cells to differentiated cells and cells treated with a PPARγ agonist. First, to compare the effects of NAFC on adipogenesis to a known differentiation stimulus, one group of cells was treated using the differentiation protocol described below and were harvested on day 8. Second, rosiglitazone (2µM, Sigma), a PPARγ agonist, was added to the media to assess PPARγ activation. Treatment media was replaced every 2 days until the cells were fully differentiated into mature adipocytes (i.e. 8 days).

Mature 3T3-L1 adipocytes

In experiments investigating the effects of commercial NA and NAFC in mature adipocytes, 3T3-L1 cells were differentiated using the following differentiation protocol: 2 days after cells had reached 100% confluence (day 0) cells received differentiation media (DM) (DMEM containing 4.5 g/L glucose, supplemented with 0.5 mM IBMX, 2µg/mL insulin, 2µM rosiglitazone and 0.25 µM dexamethasone). On day 3 differentiation media was removed and replaced with post differentiation media (PDM) (DMEM containing 4.5 g/L glucose and supplemented with 2µg/mL insulin). PDM was removed and replaced every 2

days. On day 9 differentiated cells were treated with vehicle (control), 1.25-125mg/L naphthenic acids extracted (NAFC) or Rosiglitazone (2µM, Sigma), in PDM for 48 hours. The pH of the media was adjusted to be similar across all groups using NaOH or HCl after the addition of NAFC.

Cell viability

Cells were seeded and treated in 96-well plates following the protocols described above. For the DA cells, after 8 days of treatment in cells treated in the absence of other differentiation factors 10% alamarBlue reagent (BioRad Laboratories, Hercules, CA, USA) was added to fresh media and cells were incubated for 2h. For the mature adipocytes, after 48h of treatment, 10% alamarBlue reagent (BioRad Laboratories, Hercules, CA, USA) was added to fresh media and cells were incubated for 2h. Absorbance was then read at 570nm in a plate reader (Synergy H1 microplate reader, Norgen BioTek, Thorold, ON, Canada) with a reference read at 600nm. Compounds were deemed to be cytotoxic when viability was less than 80% of the control group.

PPAR-γ ligand assay

The PPARγ ligand activity of compounds was measured using a competitive binding fluorescence-polarization PPARγ Ligand Screening Assay Kit (Cayman Chemicals, Ann Arbor, MI, USA). The PPARγ binding activity was determined for a range of concentrations of commercial naphthenic acids (CNAs)

(0.025-250mg/L; Sigma) and rosiglitazone (0.035-112.9mg/L) according to the manufacturer's instructions. Fluorescence-polarization was measured using a plate reader (Norgen BioTek) with an excitation wavelength of 485nm and an emission wavelength of 528nm. Polarization values for each compound were presented in mP units. The half-maximal inhibitory concentration ($IC_{50)}$ values were determined from a 4-parameter logistic regression. As the tested range of concentrations of CNA and NAFC did not maximally bind PPARγ, it was assumed that 10,000mg/L of each maximally bound NAFC in the 4-parameter model.

Lipid staining and quantification

To assess neutral lipid content following differentiation in DA cells, after 8 days of treatment cells were washed with DPBS (Corning), fixed with 10% formalin and stained with Oil Red O (Sigma) for 20 min, then washed 3x with water as previously described (Ramírez-Zacarías et al., 1992). The Oil Red O dye was eluted with 60% 2-propanol, and absorbance was read at 510nm (Norgen BioTek). To assess lipid accumulation following NAFC treatment in mature adipocytes, after 48 hours of treatment with NAFC, Oil red O staining was assessed as described above.

Real-time quantitative PCR

For DA cells, after 8 days of treatment, cells were washed with phosphate-buffered saline (PBS) and RNA was extracted using TRIzol reagent (Invitrogen, Waltham, MA, USA). For the mature adipocytes, after 48h of treatment, cells were washed with PBS and RNA was extracted using TRIzol reagent (Invitrogen). RNA concentrations were determined using the NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was made from 2 µg of mRNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham MA, USA) as per the manufacturer's instructions. Real-time quantitative PCR (RT-qPCR) was carried out using PerfeCTa SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD, USA) on the CFX384 Touch Real Time PCR Detection System (Bio-Rad Laboratories). The PCR cycling settings included polymerase activation (95 °C for 10 m), followed by 40 cycles of denaturing (95 °C for 15s) and annealing/elongation (60 °C for 1m). Levels of gene expression were generated using the 2(-ΔΔC(T)) method (Livak & Schmittgen, 2001) and normalized using the geometric means of two reference genes: Tyrosine 3-monooxygenase/tryptophan 5-monooxygnase activation protein zeta *(Ywhaz)* and peptidylprolyl isomerase A (*Ppia)*. Gene targets for adipocyte differentiation: peroxisome proliferator receptor activator gamma *(Pparγ)*, CCAAT enhancer binding protein beta *(C/EBPβ)*, and cluster of differentiation 36 *(CD36)*, were measured. A marker of mature adipocytes, fatty acid binding protein 4 *(Fabp4)*, was also measured. Primer sequences are in Table 1.

Statistical analysis

Data are expressed as the means +/- standard errors of the means (SEM). Statistical analyses were carried out using GraphPad Prism 9.2 (GraphPad Software Inc, San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by post-hoc testing (Dunnet multiple comparisons) when significance was indicated ($p<0.05$).

Results

PPARγ ligand activity of naphthenic acids

To determine whether there is an interaction between PPARγ, CNA, and NAFC, we performed a fluorescence-polarization based competitive binding assay using cell-free human PPARγ. CNA competitively inhibited the binding of the PPARγ-flourescent probe (FP) to PPARγ (Fig. 1). The half maximal concentration (IC_{50}) values for rosiglitazone were 2.41mg/L (8.61μM), 82.0mg/L for CNA and 269 mg/L for NAFC.

Effect of NAFC on 3T3-E1 cell viability

3T3-L1 cells treated with 125 mg/L NAFC for 8 days did not meet the threshold of 80% of the viability of cells treated with control media and thus doesn't meet the threshold for cytotoxicity. Treatment with 1.25 and 12.5 mg/L of NAFC had no effect on cell viability after 8 days of treatment (Fig. 2a).

NAFC induce adipogenesis in 3T3-L1 pre-adipocytes

To investigate whether NAFCs can induce adipogenesis, we determined lipid accumulation in 3T3-L1 cells treated with 1.25-125mg/L NAFC. There was no significant change in lipid accumulation at any dose of NAFC after 8 days of treatment in the absence of other factors inducing differentiation (Fig. 2b). Both the rosiglitazone treated cells and the cells treated with pro-differentiation compounds had increased lipid content (Fig. 2b).

To further evaluate the effect that NAFC have on PPARγ activity we measured the expression of genes that are transcriptionally regulated by PPARγ

activation namely *Pparγ, C/ebpβ* and *Cd36* as well as the marker of mature adipocytes *Fabp4* (Fig. 3). There was no significant change in *Pparγ* or *Cd36* expression at any dose of NAFC (Fig. 3 a, c, e, h), while rosiglitazone treated cells and cells treated with IBMX, insulin and dexamethasone (differentiation media) both had higher *Cd36* expression. 125 mg/L of NAFC inhibited the expression of *C/ebpβ* while cells treated with both rosiglitazone and differentiation media had higher expression of *C/ebpβ* compared to cells that were treated with media in the absence of any differentiation stimulus (Fig. 3 c, g). All other doses of NAFC had no effect on *C/ebpβ* expression. 125mg/L NAFC also induced increased expression of *Fabp4*, although to a lesser magnitude than as the rosiglitazone or differentiation media positive controls ($p<0.0001$, Fig. 3b).

Effect of NAFC on mature 3T3-L1 cell viability

None of the treatment doses of NAFC had any significant effect on cell viability (Fig. 4b).

NAFC inhibits markers of lipid homeostasis in differentiated 3T3-L1 adipocytes

To evaluate whether treatment with NAFC enhanced lipid accumulation in mature adipocytes, we differentiated 3T3-L1 cells and then treated them for 48 hours with NAFC. 1.25mg/L NAFC-treated cells had higher lipid content than control cells but this increase in lipid content was not observed at any other concentration tested (Fig. 4a,g). Next, we measured the gene markers of PPARγ activity *Pparγ, C/ebpβ* and *Cd36* as well as the marker of mature adipocytes

Fabp4 in NAFC treated mature adipocytes. We found that while there was no change in *Pparγ* expression for any groups, cells treated with 125mg/L of NAFC had significantly lower expression of *C/ebpβ, Cd36,* and *Fabp4* (Fig. 4c-f). Cells treated with both rosiglitazone and 25mg/L NAFC also had significantly lower *C/ebpβ* expression than controls (Fig. 4c).

Discussion

EDCs have been reported to affect adipose tissue development and function (Egusquiza & Blumberg, 2020; González-Casanova et al., 2020). There is evidence that OSPW and NAFC can perturb endocrine homeostasis, the effects of NAFC exposure on adipogenesis and the function of mature adipocytes remains to be elucidated (Leclair et al., 2015; Li et al., 2017; Wiseman et al., 2013). In this study we exposed 3T3-L1 cells to doses of NAFC from 1.25 mg/L to 125mg/L which encompasses the range of NAFCs reported in reference surface water sources, industrially impacted wetlands and tailings ponds (Vander Meulen et al., 2021). There is clear evidence in the literature that exposure to environmental toxicants that act as PPARγ ligands can increase adipogenesis and alter adipose tissue function (Kirk et al., 2021; Riu et al., 2011). Although both CNAs and NAFCs act as PPARγ ligands, it appears that NAFC does not bind PPARγ as strongly (Fig. 1). NAFC exposure did not cause increased lipid

accumulation in 3T3-L1 cells; an outcome that is typical of exposure to PPARγ agonists in this cell line (Zhang et al., 1996). PPARγ activation alone is enough to induce differentiation (Tontonoz et al., 1994). Similarly, we observed an induction of differentiation and lipid accumulation in cells treated with the PPARγ agonist rosiglitazone in the absence of other differentiation factors (Figs. 2a, 3). The lack of a similar effect in the NAFC treated cells argues against this mixture containing any significant PPARγ binding activity. Conversely, OSPW and the wastewater from unconventional oil and gas extraction wastewater did induce adipogenesis in 3T3-L1 preadipocytes (Kassotis et al., 2018; Peng et al., 2016). However, our findings that NAFCs did not induce adipogenesis are in line with what Peng et al. (2016) found where the non-polar fraction of OSPW containing most NAs did induce PPARγ activity in a reporter assay but did not increase lipid accumulation in adipocytes. Taken together, these data suggest that while some compounds related to oil and gas extraction can induce adipogenesis, NAFCs are unlikely to be the primary driver of this effect.

Although NAFCs did not have a significant effect on adipogenesis, there is some evidence that high doses of NAFC inhibited the transcription of PPARγ-regulated gene targets in mature adipocytes, suggesting that NAFCs may act as inhibitors of PPARγ activity in existing adipose tissue. Mature adipocytes treated for 48h with 125mg/L of NAFC had reduced expression of *Fabp4*, and *Cd36*, and treatment with both 25 mg/L and 125 mg/L of NAFC inhibited the expression of *C/ebp-β* (Fig. 4d-f). C/ebp-β is part of the group of transcription factors that,

along with PPARγ and the other C/EBP family members, is responsible for the expression of adipocyte markers in mature adipocytes (Rosen et al., 2000; Tamori et al., 2002). Inhibition of *Fabp4* and *C/ebp-β* expression is consistent with inhibition of PPARγ activity. *Cd36* expression by NAFC in mature adipocytes may result in increased lipolysis and a dysfunction in the adipocytes ability to respond to metabolic stress, and to store and release energy. Peng et al. (2016) also reported lower *Fabp4* expression in 3T3-L1 cells treated with the non-polar fraction of OSPW. Our data here also suggests that NAFC from OSPW inhibit PPARγ and may disrupt mature adipocyte function.

In summary, we have shown that exposure to NAFCs do not induce adipogenesis in 3T3-L1 preadipocytes but does inhibit PPARγ activity and the expression of genes required for adipocyte function in mature adipocytes. Therefore, these data suggest that exposure to NAFCs may have the potential to impair adipocyte function in chronically exposed animals which may impact their ability to adapt to energetic stresses and survive periods of low food availability.

Figures

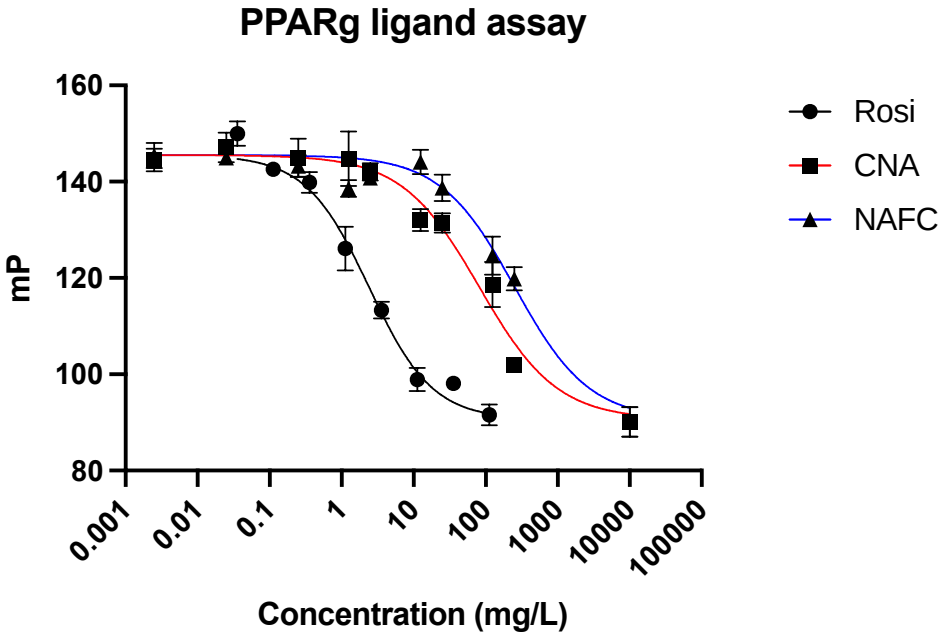

Figure 1: CNA contains PPARγ ligands
Polarization (mP) of competitive inhibition of PPARγ-fluorescent probe (FP) binding to cell free PPARγ. incubated with different concentrations of CNA and NAFC at concentrations from 0.025 mg/L to 250 mg/L and rosiglitazone (Rosi) 0.036-113 mg/L (0.1-316 µM), in the presence of PPARγ-FP.
Each value is the mean +/- SEM (Rosi n=3, CNA n=2).

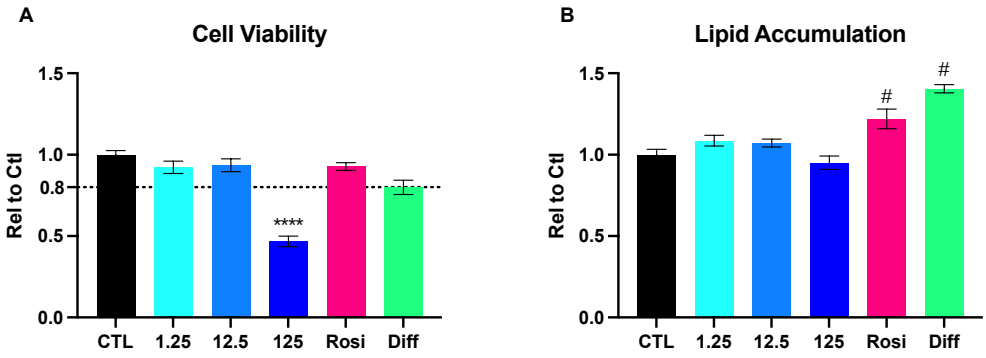

Figure 2: Effect of NAFC on adipogenesis and cell survival.
(A) Cell viability of 3T3-L1 cells treated with 1.25-125 mg/L NAFC or 2µM Rosiglitazone (Rosi) or differentiation media (Diff) for 8 days.
(B) Lipid accumulation of 3T3-L1 cells treated with 1.25-125 mg/L NAFC or 2µM Rosiglitazone or differentiation media for 8 days.
Each value is the mean +/- SEM (n=6). ****p < 0.0001, #p<0.05 positive control compared with the corresponding control group.

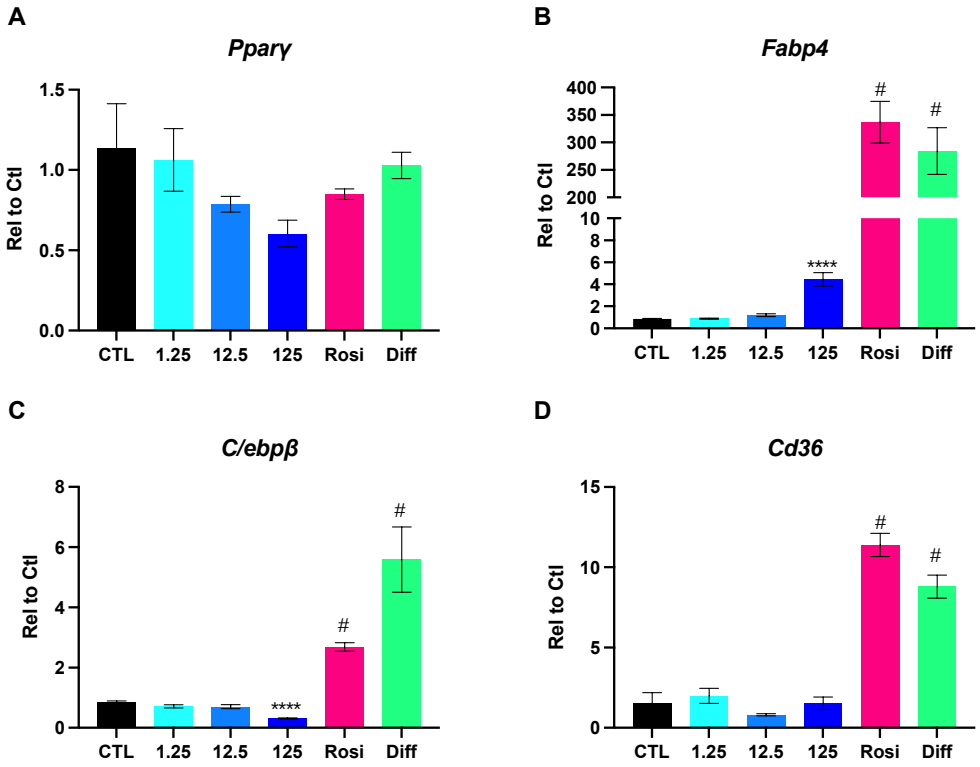

Figure 3: RNA markers of PPARγ induced adipogenesis NAFC exposed adipocytes

(A-D) Expression of adipogenic marker genes *Pparγ*, *Fabp4*, *C/ebp-β*, and *Cd36* in 3T3-L1 cells treated with 1.25-125 mg/L NAFC or 2μM Rosiglitazone or differentiation media for 8 days.

Each value is the mean +/- SEM (n=6). *p<0.05, ***p<0.001, ****p < 0.0001, #p<0.05 positive control compared with the corresponding control group.

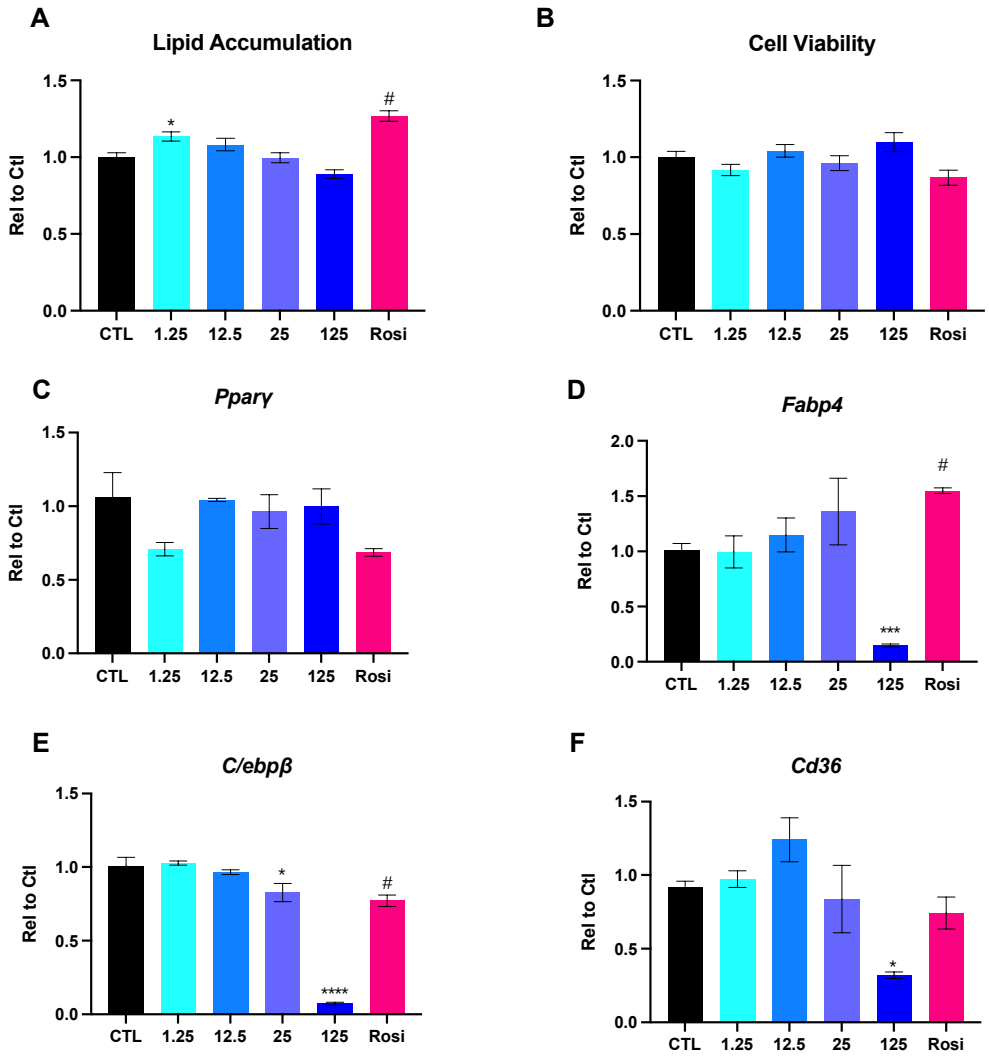

Figure 4: Lipid accumulation and markers of adipogenesis in mature adipocytes treated for 48 hours with NAFC

(A) Lipid accumulation of mature 3T3-L1 cells treated with 1.25-125 mg/L NAFC or 2μM Rosiglitazone for 48 hours.

(B) Cell viability of mature 3T3-L1 cells treated with 1.25-125 mg/L NAFC or 2μM Rosiglitazone for 48 hours.

(C-F) Expression of adipogenic marker genes *Pparγ*, *Fabp4*, *C/ebp-β*, and *Cd36* in 3T3-L1 cells treated with 1.25-125 mg/L NAFC or 2μM Rosiglitazone or for 48 hours.

Each value is the mean +/- SEM (n=6). *p<0.05, ***p<0.001, ****p < 0.0001, #p<0.05 positive control compared with the corresponding control group.

Table 1: Primer sequences.

Gene	Forward/ Reverse	Sequence	Accession No.
YwHAZ	Forward	5'-GGGGTGATTGGCAAAAGGTA-3'	NM_001356569.1
YwHAZ	Reverse	5'-CGACTTGGAAGCACAGAACT-3'	
PPIA	Forward	5'-GACAAAGTTCCAAAGACAGCAGAA-'3	NM_008907.2
PPIA	Reverse	5'-CCAAATCCTTTCTCTCCAGTGC-3'	
FABP4	Forward	5'-ATT TCC TTC AAA CTG GGC GTG-3'	NM_024406.3
FABP4	Reverse	5'-CTT TCC ATC CCA CTT CTG CAC-3'	
PPARy	Forward	5'-GCG GAA GAA GAG ACC TGG G-3'	NM_001127330.2
PPARy	Reverse	5'-GTG ACT TCT CCT CAG CCC G-3'	
CD36	Forward	5'-CTGTGTCCTTCTGAATCATTTAACC-3'	NM_001159558.1
CD36	Reverse	5'-TTCAGTCTCACACTGCTGCTAT-3'	
C/EBP-β	Forward	5'-CCG TGG TGG TTT CTC GA-3'	NM_001287739.1
C/EBP-β	Reverse	5'-TTT TTG CTC CCC CTA CTCC GG-3'	

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Declaration of Competing Interest
None.

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4. Chapter 4: Discussion

There is concern that chemicals released into the environment from oil sands mining activity in the AOSR are impacting the health of exposed wildlife and is contributing to dwindling population of indigenous species (Bakx & Normand, 2018). It has been observed that the baculums of male *Lontra canadensis* are weaker in animals from areas with high amounts of oil sands activity than areas in the AOSR that are unaffected by oil sands activity(Thomas et al., 2021). This observation may be a mechanism contributing to the lower otter fertility (Gutgesell et al., 2019; Raez-Villanueva et al., 2019) because adequate baculum strength is required for successful reproduction (Stockley et al., 2013). The production and storage of OSPW required by bitumen extraction in the AOSR is one of the primary sources of PRCs in the environment and the toxicity of OSPW is considered to be primarily due to polar organic constituents, including NAFCs (Bartlett et al., 2017). Adipocytes and osteoblasts are two different MSC derived cells that exist in bone, the focus of this thesis is on the effects of NAFCs from OSPW on path selection during differentiation of MSC-derived progenitor cells toward osteoblasts generating cortical bone tissue, or adipocytes contributing to BMAT, two important contributors to bone quality and strength.

Bone adapts to changes in loading and repairs microdamage throughout life through the process of remodeling, a tightly regulated process that involves resorption of old bone tissue and the formation of new cortex material.

Osteoblasts deposit new cortex material as they differentiate before terminally differentiating into osteocytes or undergoing apoptosis. Our finding that exposure to NAFC inhibits the deposition of ECM at doses of 12.5mg/L and higher provides evidence that NAFC can negatively affect bone formation. We observed increased expression of markers of GR activity with NAFC exposure; however, future experiments treating 3T3-E1 pre-osteoblast cells with a GR antagonist prior to NAFC exposure would be needed to determine whether GR activity is required for the inhibition of osteoblastogenesis caused by NAFC. We also observed that NAFC lowered ECM production to a much greater extent than the GR agonist dexamethasone and is therefore likely inhibiting osteoblast differentiation through other pathways in addition to GR. Although not investigated in our study, NAFCs are well known to induce aryl hydrocarbon receptor (AHR) activity (Marentette et al., 2017), which is primarily responsible for initiating xenobiotic metabolism in response to environmental chemicals. However, AHR receptor activity has also been shown to induce a pro-aging phenotype in many tissues and impair mitochondrial function (Brinkmann et al., 2020). The deposition of ECM during bone formation is a very energy intensive process. If NAFC exposure impairs mitochondrial function in osteoblasts this could be a plausible mechanism that explains, in part, the inhibition in ECM deposition following NAFC exposure. There are several other pathways by which NAFCs may inhibit osteoblast differentiation including Wnt/Notch, PTH and bone morphogenic proteins (Rutkovskiy et al., 2016). Given the complexity and number of factors

influencing osteoblast differentiation, it may be worthwhile to use -omics techniques to capture a global view of the transcriptional response of osteoblasts to NAFC exposure.

The observation that NAFC inhibit osteoblast differentiation and ECM deposition support our main hypothesis that NAFCs are altering MSC progenitor cell differentiation to impair bone formation. The second avenue by which NAFCs may alter MSC progenitor cell differentiation that we investigated is whether NAFC exposure drives progenitor cells toward a pro-adipogenic phenotype and therefore promotes bone marrow adipogenesis.

Bone marrow adipogenesis involves proliferation of adipocytes in the medullary cavity of long bones and is associated with lower bone strength (Rosen & Bouxsein, 2006). The metabolic function of bone marrow adipose tissue is unknown, however, increases in marrow adiposity are observed in a variety of metabolic diseases and aging (Sebo et al., 2019). In mice, the PPARγ activating drug rosiglitazone induces increased bone marrow adipogenesis (Lu et al., 2016), which given previous reports that OSPW contains PPARγ ligands (Kassotis et al., 2018; Peng et al., 2016) led to the hypothesis that components of OSPW may induce marrow adipogenesis. However, we subsequently found that while commercial NA mixtures do contain PPARγ ligands, NAFCs did not induce adipogenesis. These data did not support our primary hypothesis that NAFC shifted MSC progenitor cell differentiation away from an osteoblast phenotype towards an adipose phenotype. While NAFC exposure did inhibit osteoblast

differentiation, it did not induce adipocyte differentiation. In this same study where we investigated whether NAFC induced adipogenesis we also investigated the effects of NAFC in mature adipocytes. We found that the binding of components in NAFCs to PPARγ are likely antagonistic and inhibit the expression of enzymes required for the regulating lipolysis and the insulin response in adipocytes. These effects are not isolated to bone marrow adipocytes and are likely unrelated to the impact that NAFCs may have on bone strength. However, they do suggest that NAFCs may impair adipose tissue function in exposed animals.

The work presented in this thesis is the first to describe the direct effects of NAFCs on bone cells and pathways affecting osteoblast differentiation, and the first to assess the effects of isolated NAFCs on adipogenesis. In mammals where the baculum is required for successful reproduction, the inhibition of remodeling or formation of the baculum bone by NAFCs or other PRCs would have negative consequences for the reproductive health of these animals. Further, as we have shown that NAFCs can affect glucocorticoid signaling which is involved in numerous physiological process that are essential for life. Therefore, exposure to NAFCs may have a greater impact on the health of mammals than previously thought. This has broad implications for the health of ecosystems in areas affected by oil sands activity like the AOSR.

4.1. Limitations and Future directions

The data presented in this thesis demonstrate potential toxicologic effects of NAFCs on bone and adipose tissue development and metabolism. These experiments were *in vitro* and future experiments investigating whether the observed effects on osteoblasts and adipocytes translate to lower bone strength and impaired bone remodeling in adult animals, and impaired adipose tissue metabolism *in vivo*. Future studies should investigate the effects of NAFCs on bone formation, and in particular baculum formation, *in vivo*, as osteoblast differentiation is necessary for skeletal development (Rutkovskiy et al., 2016). The concentrations of NAFCs used in these experiments was based on the concentrations reported in environmental samples, however it is unknown what the concentration of NAFCs are in exposed animals. This is a limitation for the study of the toxicity of NAFC in general, which is complicated by the highly complex nature of NAFC mixtures and the lack of analytical techniques presently available to robustly measure NAFC concentrations in tissue samples (Brown & Ulrich, 2015). Thus, all studies of the toxicity of naphthenic acids in animals will need to estimated exposures concentrations based on levels measured in bodies of water in the environment as we have done in the work presented in this thesis. As analytical techniques are developed to be able to identify tissue concentrations of NAFCs in wildlife, toxicology and pharmacokinetic studies of the effects of NAFCs should be revisited to get a more accurate understanding of what the effects of real-world concentrations of NAFCs are on cells and animals. Another

limitation of the work presented in this thesis is that we observed an induction of markers of GR activity with NAFC treatment, however, we did not determine that these were the sole mechanisms by which NAFC inhibits osteoblast differentiation and ECM deposition. Future studies could use a knock-down of the GR gene or treatment with a selective GR antagonist like RU-43044 prior to treatment with NAFC and see whether the observed effect on ECM deposition is attenuated (Clark, 2008).

In conclusion, the work presented in this thesis presents a mechanism by which NAFCs may impair bone strength and metabolism in exposed animals. Our findings support the hypothesis that exposure to NAFCs alter the differentiation of MSC progenitor cells to favour reduced bone formation. The data presented in this thesis provides evidence that; 1. NAFCs inhibit the differentiation and deposition of ECM of osteoblasts and act, in part, through the GR and 2. NAFCs do not induce adipogenesis of adipocyte precursor cells but rather inhibit PPARγ target gene expression suggesting NAFCs may impair key adipocyte functions in whole body energy homeostasis. Furthermore, this evidence can direct future *in vivo* experiments investigating the effects of NAFC on both bone strength and adipose tissue metabolism, both of which are fundamental to the survival of wildlife.

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