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Embryonic Exposure to Harm Reduction Tobacco Products Cause Mitochondrial and Behavioral Defects Similar to Conventional Tobacco

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by

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ABSTRACT OF THE DISSERTATION

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Master of Science, Graduate Program in
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Developmental toxicology is an ever-changing field dedicated to ensuring the safety and efficacy of foods, drugs and chemicals available to the public, specifically concerned with effects on a developing embryo. While many studies have uncovered the detrimental health effects of tobacco use, a concern to the health problems associated with harm reduction tobacco products (HRTPs) grows as the usage increases, specifically in women who are, or may become pregnant. Using a novel approach analyzing mitochondrial network morphology called Mitochondrial Network Analysis (MiNA), significant changes in mitochondrial network morphology were found in human embryonic stem cells (hESCs) exposed to both Camel and Camel Blue cigarettes, conventional and HRTP respectively. Supplementation with ascorbic acid led to a general improvement of network morphology for both products suggesting ROS as one mechanism of toxicity. To

determine if prenatal exposure to Snus, a non-combustible HRTP, causes any increased risk of neurological damage such as anxiety and motor control, pregnant mice were injected with a Snus tobacco extract, and allowed to age into adulthood. The adult mice were then tested for such neurological issues using the elevated plus maze (EPM) and the Suok test. Results suggest female mice are more susceptible to increased behavioral issues such as anxiety and reduced motor control when exposed *in utero* to Snus tobacco extract than male mice compared to the untreated. This suggest that these neurological concerns may be linked in some way to the X-chromosome, and environmental toxicants such as tobacco may increase chances of occurrence. These studies together suggest that embryonic exposure to HRTPs may lead to other increased risks, not typically evaluated, that may be equal or more detrimental than conventional tobacco and must be taken into consideration when making claims of reduced harm.

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1 Chapter

Introduction to the Thesis

1.1 Bone formation *in vivo*

Given the complexity of our skeleton, it is clear that if the process of bone development, osteogenesis, were to be misregulated, several devastating abnormalities and diseases can occur. This is the case with many congenital and degenerative diseases which can be fatal or cause dramatic loss in quality of life. To complicate researchers understanding of these diseases, our bones arise from two embryonic origins, mesoderm for our axial and appendicular skeleton, and cranial neural crest (CNC) cells which give rise to the majority of the craniofacial bones (Sera and zur Nieden, 2017). And while these two sources for bone begin very differently, their lineages meet at a common cell type, the mesenchymal cell, and from there on are indistinguishable from one another. However, the process in which these cells produce bone is different depending on the type of bone being produced. Long bones, such as the femur, use a process called endochondral ossification, which requires a chondrocyte intermediate to build a scaffold for the builders of the bone, osteoblasts (Mackie et al., 2008). Flat bones, such as those found in the skull, do not require an intermediate stage, and can directly produce osteoblasts for depositing the bone matrix, called Intramembranous ossification (Franz-Odendaal, 2011). The differences in these processes contributes to the

variety of defects and diseases which can affect the process of bone formation and homeostasis. In fact, both bone development and homeostasis involve constant remodeling of the matrix, using osteoclasts to destroy areas of the bone, and new osteoblasts forming to re-build it. These specific areas of bone remodeling are known as basic multicellular units (BMU) and are estimated to remodel the entire skeleton every 2 to 4 years (Whyte, 2006).

1.2 Congenital anomalies in the skeleton

1.2.1 Mesodermal Bone Diseases

The majority of the skeleton, with exception to most craniofacial bones, arises from the mesodermal germ layer. Due to the complex gene regulatory networks and differentiation process throughout osteogenesis, a number of diseases can arise which lead to complex fractures, density and growth defects, and even death. Even without disease or developmental defects, by 2025, more than 3 million fractures are predicted to occur each year in the United States (Burge et al., 2007). Hip fractures, while rarely occurring before the age of 70, are significantly rising throughout the world (Baron et al., 1996). It is estimated that by the year 2050, 6.26 million people a year will have a hip fracture occur. These fractures lead to an increased risk of death, and typically requires prolonged disability and a potential need for institutionalization. However, it was estimated that in 1990, 1.66 million hip fractures occurred in people under the age of 35, 72% of which were women (Cooper et al., 1992).

Many of these hip fractures are caused by osteoporosis, which is estimated to affect more than 14 million people in the United States by the year 2020 (Burge et al., 2007). Osteoporosis is characterized by porous and weak bones which lead to increased fracture incidences, some of which can be life threatening such as with the hip. In the United States, 7% of individuals who survive from fractures have some permanent disability, and 8% require prolonged institutional care. Unfortunately, approximately 31,000 deaths occur within 6 months every year from hip fractures (Harvey et al., 2010). However, osteoporosis does not just affect the hip, injuries are also prevalent in the spine and wrist. The attributed loss of bone mass and matrix architecture deterioration occurs in both men and women, typically around age 40, however within the first 5-10 years from the onset of menopause, women have an increased risk of developing osteoporosis (Lindsay, 1988). Osteoporosis not only burdens the affected individual and their family, but also greatly affects the economy. In 2005, osteoporosis in the United States led to over 2 million fractures costing $17 billion in direct medical costs (Burge et al., 2007). While this disease is consistent phenotypically, there is no single cause. Primary osteoporosis is divided into type I, which is due to sex hormonal imbalances, and type II which typically occurs from old age and improper nutrition. Secondary osteoporosis can occur from problems in the endocrine, gastrointestinal, and rheumatological systems, as well as from cancer and substance abuse (Snaith, 1999). Treatments for osteoporosis vary depending on the individual cause, however estrogen replacement therapy (ERT),

supplementation of bisphosphonates, calcitonin, sodium fluoride, calcium and vitamin D have all been used to treat and prevent bone loss. Other treatments include anabolic therapy and PTH/PTHrP injections, which help to prevent fractures, however the efficacy of these compounds is not well established (Dempster et al., 1993; Reeve et al., 1980; Stewart, 1996).

A similar manifestation of bone fragility occurs in patients with osteogenesis imperfecta (OI), also known as brittle bone disease. This congenital disease, which occurs in one out of 15,000-20,000 births (Forlino and Marini, 2016), causes low bone density and increased risk of fractures; however, it can also have a damaging effect on other organs (Morello, 2018). Depending on the severity of the skeletal deformation during development and birth, impaired pulmonary and respiratory function and loss of hearing have occurred in OI patients (Forlino and Marini, 2016). OI is also comparable to osteoporosis in that the causes of this disease are also heterogenous, but similarly effect the extracellular matrix and its ability to mineralize and aid in matrix-to-cell signaling. The most commonly affected gene which contributes to OI is type 1 collagen, and other proteins related to its synthesis (Morello, 2018), due to its prominence in the extracellular matrix. In addition to type 1 collagen defects, CREB3L1, SP7, and WNT1 have also been implicated in impaired osteoblast differentiation, leading to OI. Of these three, WNT1 is most prominent due to its role in the canonical Wnt pathway, which lets b-catenin enter the nucleus, activating expression of genes necessary for osteogenesis to occur (Forlino and Marini, 2016). Due to OI being a congenital

disease, treatment must start early, and typically starts with receiving nitrogen-containing bisphosphonates, which help to inhibit future bone resorption by forcing apoptosis in osteoclasts (Luckman et al., 1998). Two recent therapies which are being tested include pharmaceuticals which inhibit RANKL, leading to inactivation of osteoclasts (Bone et al., 2017; Hoyer-Kuhn et al., 2015), or inhibition of cathepsin K (Langdahl et al., 2012; Rizzoli, 2016), a lysosomal protein produced in osteoclasts for matrix digestion. However, these treatments must be started immediately, and will require a multidisciplinary treatment including physiotherapy, occupational therapy, and surgery for correction of some deformities. A clinical trial published in 2017, tested whether whole body vibration (WBV) therapy, which was found to improve bone volume in an OI mouse model, would improve bone mass or density in children with OI. Unfortunately, this study showed that WBV had no improvement to bone mass after 5 months of this therapy (Vanleene et al., 2013; Högler et al., 2017).

Another disease associated with aggressive osteoclast bone resorption occurs in patients with Paget's disease, and is followed by improper osteoblast repair (Whyte, 2006). Paget's disease is the second most common bone disease (Roodman et al., 2005), occurring in approximately one in 100 people over the age of 40 in the United States, and typically affects only one of a few bones, most often the skull, pelvis, vertebrae, femur, and tibia (Whyte, 2006). Treatment of this disease is directed at inhibiting osteoclast formation, resorption, or inducing apoptosis. While pharmaceuticals targeting inhibition of osteoclast may be helpful

for OI and Paget's disease, osteopetrosis, also known as marble bone disease (MBD) would require the opposite treatment. MDB is also a congenital heterogenous disorder of bone remodeling, in which osteoclasts function is decreased, and affects one in 20,000-500,000 births, depending on the dominant or recessive mutation (Dahl et al., 1992; Tolar et al., 2004). This causes the bones to become fragile due to remodeling of primary woven bone to lamellar bone (Fudge et al., 2007). While long bones seem to be most affected, some neurological issues such as blindness, deafness, facial paralysis, and even mental retardation have occurred (Fudge et al., 2007; Lam et al., 2007). While some patients develop an adult form of osteopetrosis, infantile osteopetrosis is most often fatal if untreated within the first decade due to hemorrhage, pneumonia, anemia or sepsis (Lam et al., 2007). However, regardless of etiology, pharmaceuticals are commonly used for the treatment of most osteogenic diseases which are designed to affect the balance of bone building and resorption.

1.2.2 Cranial Neural Crest Bone Diseases

While the previous diseases mentioned affect the axial and appendicular skeleton, derived from the mesodermal lineage, there are several craniofacial diseases and defects which arise due to improper formation or differentiation of CNC cells. Between the 3rd and 8th week of pregnancy, complex developmental and organizational processes occur in order for proper craniofacial development (Trainor, 2010). The most commonly treated congenital craniofacial defect is cleft lip/palate, occurring in 1-7 per 1000 births in the United States (Bernheim et al.,

2006; Hooper et al., 2007; Mossey et al., 2009). Cleft lip/palate can lead to death in extreme cases, or where proper care and medicine is unavailable, but it can also lead to an enormous burden on quality of life due to effects on speech, hearing, appearance, and psychological impacts on patients (Mossey et al., 2009). Cleft lip/palate occurs when CNC cells improperly migrate and develop, leading to incomplete fusion of the nasal and maxillary processes on each side of the head (Sperber, 2002). Mutations in multiple genes have been contributed to causing cleft lip/palate such as fibroblast growth factor (FGF), sonic hedgehog (SHH), bone morphogenic proteins (BMPs), and others (Rice et al., 2004). However, some environmental factors such as retinoic acid, thalidomide, and maternal smoking and alcohol use has also been found to contribute to cleft lip/palate (Mossey et al., 2009). In fact, over 450 mendelian disorders are associate with cleft/lip palate, making this disease specifically interesting to study (Fryns and de Ravel, 2002). Treatment of these patients typically involves surgical treatment, feeding counseling, hearing and speech assessment as well as significant orthodontic treatment (Vlahovic and Haxhija, 2017).

Another disorder commonly associated with improper development and migration of CNC cells is Treacher Collins syndrome (TCS), which occurs in one in 50,000 births (Chang and Steinbacher, 2012). TCS mostly arises from defects in the first and second pharyngeal arches and can lead to multiple defects such as a smaller nose, jaw ears, as well as development of cleft lip/palate (Trainer, 2010, Chang et al., 2012). Additionally, hearing is often affected, as well as defects in brain

development such as microcephaly, and causing mental retardation and psychomotor delay (Phelps et al., 1981; Milligan et al., 1994; Cohen and Krelborg, 1992; Teber et al., 2004; Trainor, 2010). While TCS expresses a variance of phenotypes, it is caused by an autosomal dominant mutation of the TCOF1 gene leading to a loss of protein function (Wang Jabs et al., 1991; Dixon et al., 1991). Although only one gene seems to be affected, the range of phenotypes likely arises from the over 50 different mutations found in the TCOF1 gene have been linked to TCS (Splendore et al., 2000). Of most concern when treating this disease is the difficulty of breathing and feeding directly after birth, which may require emergency tracheostomy. During the 1st year, additional surgeries may be helpful to correct some deformities and will require oral, ocular, dental, pediatric, and craniofacial specialists (Trainor, 2010).

Another major craniofacial disease associated with a single gene is Crouzon syndrome. This less common disease occurs in 16.5 in 1,000,000 births (Cohen and Krelborg, 1992), and is an autosomal dominant disease associated with fibroblast growth factor receptor 2 (FGFR2) (Wen et al., 2010; Lin et al., 2017; Fan et al., 2018). Crouzon syndrome manifests as severe craniofacial deformities such as premature fusion of one or more cranial sutures, ocular disorder and midfacial hypoplasia (Khominsky et al., 2018). In addition, individuals with Crouzon syndrome are at risk of premature fusion of the spheno-occipital synchondrosis (SOS) (Driessen et al., 2017), a cartilaginous structure between the upper mandible and the occipital bone. While surgeries may help improve certain

functions, there are severe risks of complications, and many patients are still unable to lead normal lives even after therapy (Stavropoulos et al., 2011).

1.3 The Impact of Toxicology

While the diseases listed above often stem from one or more genetic causes, environmental exposure to chemicals may also alter the normal process of osteogenesis to lead to brittle or over-mineralized bone. The study of toxicological compounds is greatly responsible for the increasing general health, safety, and lifespan of humans, with regard to skeletal health specifically, but also generally. While the toxicity of compounds found in nature has been of interest since the early Greek mythologists and likely earlier, the first major contribution to the field came from the Spanish chemist and physician Mathieu Orfila, known as the father of toxicology, who wrote “Traite Des Poisons” in 1814. In his book, Orfila describes not only the properties of compounds, but also how they affect tissues, and treatments for a long list of toxins such as mercury, arsenic, acids, ammonia, phosphorus (Orfila, 1814). The passage of the Pure Food and Drugs Act in the United States, which became known as the FDA, enabled the federal government to investigate and enforce illegal drug manufacturing and distribution, as well as any foods, which were unsanitary or have had the addition of ingredients, which posed a threat to health (59th Congress, 1906). Over 100 people died from a therapeutic named “Elixir of Sulfanilamide”, which had become a popular treatment for streptococcal infections. The company that sold this elixir had been dissolving

the sulfanilamide in diethylene glycol (DEG), which is poisonous and the main component of antifreeze (Ballentine, 1981). The incident caused a major push from the public for the FDA to be more proactive and ensuring the safety of drugs marketed in the US. In 1953, thalidomide was synthesized and grew to be a popular drug for morning sickness associated with pregnancy (McBride, 1961), and by 1958 was being marketed worldwide. Obstetrician William McBride noticed that a high rate of women using thalidomide were giving birth to children with major developmental defects including shortened or missing limbs at a rate of 20% when the typical rate of congenital abnormalities at the time was 1-5% (Vargesson, 2015). During this time, for drugs to be passed, they did not have to be tested for causing developmental defects in pregnant animals. Therefore, because thalidomide was found to not cause fatal overdose, the drug was cleared for distribution. The births of an estimated 10,000 children affected by thalidomide caused a major shift in the practice of toxicology to include pre-natal screenings known as developmental toxicology (Kelsey, 1988).

1.4 Toxicity of tobacco

The most common unwanted environmental exposure that may elicit toxicity is tobacco smoke, In 2003, the Center for Disease Control and Prevention (CDC) estimated that tobacco usage caused serious medical issues to 8.6 million people in the U.S. (Department of Health and Human Services, 2014); Worldwide however, the World Health Organization estimates that 8 million people die

annually either from directly smoking or from environmental smoke making tobacco one of the most dangerous legal products worldwide (World Health Organization, 2019). Tobacco has been used culturally for thousands of years, however modern cigarettes and other tobacco products include numerous additives, which according to patents give flavor, preserve the tobacco, or give the product distinct properties (Krüsemann et al., 2018; Keaton, 1961; Reid, 1981). Unfortunately, many of the additives have been found to be toxic (Talhout et al., 2011; Pagumartten et al., 2017) and yet due to the addictive properties of nicotine consumers will willingly accept the health risks (Balfour, 2002). A major complexity of identifying the specific toxic agents within tobacco smoke lies with the process of combustion, whereby roughly 9,000 different compounds are produced (Pagumartten et al., 2017).

There are three main components of tobacco smoke, mainstream, side-stream, and third hand smoke. Mainstream smoke is directly inhaled by the user, which comes through the interior of the cigarette, while side-stream smoke comes off the end of the burning cigarette. Sidestream smoke is one of the components of secondhand smoke while another component of second-hand smoke is produced when the smoker exhales. The exhaled smoke contains a different profile of compounds than the side stream smoke due to the absorption in the lungs (McAughey et al., 1994; Moldoveanu et al., 2007; Garey et al., 2004). Third hand smoke, in turn, refers to compounds and particulate matter produced from the smoke, which deposits onto surfaces such as the carpet, drapery, and furniture.

Third hand smoke is specifically dangerous because it primarily effects young children, who are crawling on the floor and touching their mouths. Over long periods of time the compounds can become more toxic through oxidation (Ferrante et al., 2013; Merritt et al., 2012; Escoffery et al., 2012; Sleiman et al., 2010a). Additionally, nicotine found in tobacco smoke can react with residual nitrous acid on the surfaces of carpets and furniture, forming carcinogenic tobacco-specific nitrosamines (TSNAs) which are strong carcinogens (Sleiman et al., 2010b).

Due to a growing public concern regarding the incidence of lung cancer and smoking, American tobacco companies began producing “light” cigarettes, which were marketed as safer (Backinger, 2007). These new cigarettes reportedly contained lower tar and nicotine than conventional cigarettes, as well as having better filters and paper. The marketing worked, and by the 1980’s “light” cigarettes outsold conventional cigarettes (Moran, 2015). A study by Hsu and Grodal found that between 1964 and 1993, nicotine and tar levels continually increased to 74% and 7% respectively (Hsu and Grodal, 2015). In 2009, with over 400,000 deaths a year from tobacco smoke in the U.S., the FDA banned the tobacco industry from marketing cigarettes as “light” or having reduced harm (111th Congress, 2009).

Light cigarettes were not the only tobacco products thought to be less harmful. Non-combustible tobacco products such as chewing tobacco and Snus have also been considered less harmful than conventional cigarettes due to the absence of smoke and other carcinogens associated with combustion (Hatsukami et al., 2004; Gartner et al., 2007). However, while chewing tobacco and other non-combustible

oral tobacco products are free from certain toxins, their consumption is also associated with high rates of oral cancer, throat cancer, gum disease, and other oral complications (Critchley and Unal, 2003; Weintraub and Burt, 1987).

In the early 2000's a Chinese pharmacist created the first electronic cigarette, which used electricity to vaporize a liquid comprised of nicotine diluted in a propylene glycol solution (Bekki et al., 2014), however in 1963 a U.S. patent was accepted for a "Smokeless non-tobacco cigarette" (Gilbert, 1963). By 2007 e-cigarettes were introduced to the American market, by 2016 the e-cigarette market became a $7.1 billion industry in the U.S., and by 2024 is estimated to hit $44.55 billion (Hexa Research, 2017). E-cigarettes grew popular due to an expansive list of sweet and savory flavors, as well as the culture of vaping, which included modifying the vaporizers to create larger and denser clouds of vapor (Jarmul et al., 2017). The list of flavors, which includes bubblegum, strawberry and flavors called "unicorn poop" enticed children and teens who wanted to experience these flavors and join into the culture (Jackler and Ramamurthi, 2017). Similar to chewing tobacco and other non-combustible tobacco products, e-cigarettes have been marketed as safer because of the reduced additives and absence of smoke. However, because of the huge rise in e-cigarette users, many researchers have directed their focus to studying the health consequences of the vapor, which has not been thoroughly explored.

1.5 Developmental Toxicity

Developmental studies in the 1980's determined that women who smoke tobacco during pregnancy increase the risks of their babies having premature births, low birth weight, developmental defects and increased risk of mortality (Stein and Kline, 1983; Kleinman and Madans, 1985; Christianson, 1980; Naeye and Peters, 1984). Prior to modern day screenings, toxicity screens used to only involve testing on animals to determine the limits at which the drug or chemical was lethal. The use of animal testing is a controversial subject but has been the prominent method of toxicity testing until only recently when the Organization for Economic Co-operation and Development (OECD) set international guidelines for the testing of chemicals. The establishment of mouse embryonic stem cells (ESCs) from isolated mouse blastocysts led to a major a breakthrough in toxicity screening (Evans and Kaufman, 1981). *In utero*, ESCs are pluripotent meaning they possess the ability to differentiate into any cell type found in the adult body. In 1997, using the ability to culture and differentiate mouse ESCs into beating cardiomyocytes, a method to determine embryotoxicity called the embryonic stem cell test (EST) was published (Spielmann et al., 1997). To determine whether a chemical substance is embryotoxic, the EST uses only three endpoints: the cytotoxic effect on both mouse ESCs and 3T3 fibroblasts, and the inhibition of ESC differentiation into cardiomyocytes (Spielmann et al., 1996). The inhibition of cardiomyocyte differentiation reflects the ability of the fetal cells forming heart muscle *in utero*, while the toxicity of the ESCs and the 3T3 fibroblasts help to determine whether

the fetal cells are more or less susceptible to toxicity from a specific compound than the maternal cells. Performing this test on a range of doses also allows for the computation of concentration-response curves to determine a half-maximal inhibitory dose (IC_{50}). An important feature of this protocol is that it uses two established cells lines, embryonic stem cells of the mouse cell line D3 (Doetschman et al., 1985), and differentiated fibroblast of the 3T3 cell line (Noda et al., 1986), therefore reducing the number of sacrificed animals.

1.6 Human Embryonic Stem Cells

While testing on human embryos is illegal, the culturing of human embryonic cells has allowed for better determination of health consequences associated with involuntary embryonic exposure. Because developmental toxicity requires the study of embryo development, the ability to culture human stem cells, which are able to differentiate into all cells found in the developing embryo, has given scientists a tool to study how specific compounds may affect embryo development. Isolation of the first human ESC (hESC) line, H9, was established through culturing donated cleavage-stage human embryos and allowing them to develop *in vitro* until the blastocyst stage (Thomson, 1998). At the blastocyst stage, the inner cell mass is isolated from the trophectoderm using immunosurgery and allowed to expand on a mouse fibroblast feeder layer in Dulbecco's modified Eagle's medium, supplemented with 20% fetal bovine serum, glutamine, β-mercaptoethanol and nonessential amino acids. Prior to the establishment of defined hESC growth

media such as mTeSR™ (STEMCELL Technologies), mouse ESCs maintained pluripotency using a mouse fibroblast feeder layer. The feeder layer secretes leukemia inhibitory factor (LIF) into the medium, which signals to the cells to maintain their undifferentiated state through activation of the Janus Kinase-signal transducer and activator (JAK/STAT) pathway (Frye et al., 2003). Therefore, the supplementation of LIF into the media can maintain pluripotency without the need for a feeder layer, however, hESCs do not require the use of LIF (Humphrey, 2004), and are instead maintained through a more complex network of pathways including the SMAD2,3 pathways via activation by Activin A or Nodal (Beattie et al., 2005; Xiao et al., 2006; Xu et al., 2008), as well as the Wnt and mitogen activated protein kinases (MAPK) pathways (Sato et al., 2004; Ohtsuka and Dalton, 2008; Dalton, 2013).

1.7 Human ESCs in toxicity screening

Multiple major publications gave new techniques on using human ESCs to determine embryotoxicity as well as drug discovery and efficacy (Jensen et al., 2009; Krug et al., 2013). A study from our lab found that ESCs from non-human primates such as the common marmoset and the rhesus monkey did not respond to embryotoxic compounds in the same manner as rodent ESCs (Walker et al., 2015), suggesting human cells will also respond differently. In 2010, a paper was published discussing the efficacy of using hESC derived neural progenitor cells (NPCs) as a tool for studying developmental neurotoxicity (Breier et al., 2010).

Another article that year argued hESCs are a good model for male reproductive toxicity screening (Krtolica and Giritharan, 2010), highlighting the idea that hESCs can be used for a variety of developmental screening. To determine whether new drugs would have cardiotoxic effects, hESC-derived cardiomyocytes were used to get dose response curves of the extracellular field potentials when exposed to 12 cardiac and non-cardiac drugs. They found this method to be predictive of responses found during clinical trials and suggested that this model should be used in addition to other preclinical trials (Braam et al., 2010). Additionally, a method for studying hepatotoxicity was developed using hepatocyte derived from human pluripotent stem cells, and similarly found that high efficacy with similar results to primary hepatocytes (Medine et al., 2013). Previously, a study in which our lab collaborated on, discussed the efficacy of video bioinformatics on hESC colonies exposed to different chemicals over time. Video bioinformatics was able to provide relevant information to toxicity and embryotoxicity such as the changes to colony size and the kinetics of their differentiation into osteoblasts (Talbot et al., 2014).

1.8 Tobacco toxicity to developing cells/embryos

To determine the embryotoxic effects from exposure to tobacco and nicotine, studies have used both ESCs as well as animal models such as zebrafish and chicks. Zebrafish, being a low cost and quick reproductive cycle model, can be used to screen large number of toxicants (Tan et al., 2011; Wiley et al., 2017). One such study assessed acute, developmental, cardiac, and neurotoxicity in

zebrafish larvae exposed to condensate from tobacco smoke of various cigarette types. They found that exposure to different cigarettes led to an array of defects such as having a curved body profile, cerebral hemorrhaging, and changes to heart rate and melanocyte patterning in a dose dependent manner (Ellis et al., 2014). A similar study assessed the developmental effects of both cigarettes and e-cigarettes and found that both led to severe heart malformation, pericardial edema and reduced heart function in zebrafish in a dose dependent manner. This was coupled with a cardiac differentiation of hESCs exposed to both cigarette types, finding a delay in transition through mesoderm accompanied by reduced expression of cardiac transcriptions factors (Palpant et al., 2015). Using chicken embryos, a study found that exposure to sidestream whole smoke solutions led to a drastic reduction in embryonic movements (Ejaz and Woong, 2006), which has been correlated to increased risk of fetal mortality in pregnant women (Leader et al., 1981; Valentin and Maršál, 1987; Moore and Piacquadio, 1989). However, the use of animal models can still be effective for studies, which cannot be done on human ESCs such as behavioral studies. Additionally, the use of stem cells does not completely recapitulate an embryo growing in the uterus to term, and therefore ESC research cannot be used to completely asses toxicity and teratogenicity.

1.9 Osteogenic differentiation

1.9.1 Adult Stem Cell Differentiation

While the establishment of ESC research led way to complex studies into osteogenic and other tissue developmental processes, prior to the commonplace of ESC research, researchers had been studying adult tissues *in vitro* to help determine how osteogenesis occurred *in vivo*. During the late 60's researchers had begun to understand that intramembranous osteogenesis occurred first through laying down a complex structural extracellular matrix prior to mineralization (Marvaso and Bernard, 1977; Anderson, 1969; Bonucci, 1969). Then, the first mammalian osteogenic *in vitro* differentiation was produced using tissue dissected from mouse calvaria mesenchyme using media containing ascorbic acid (AA) (Marvaso and Bernard, 1977). However, this differentiation was not robust or consistently reproducible. Due to the understanding that alkaline phosphatase plays a role in mineralization, another group cultured mesenchymal cells from mouse periosteum with β-glycerophosphate (BGP), which was far more consistent and robust than previous attempts at osteogenic differentiation *in vitro* (Tenenbaum and Heersche, 1982). This landmark in osteogenic research enabled the field to push further into understanding how bone develops *in vivo*.

During this time, it was also hypothesized that bone marrow contained progenitor cells that gave rise to bone. It was shown that bone marrow stromal cells, isolated from rate femurs, were able to form mineralized nodules when cultured with AA and BGP, in addition to dexamethasone which was found to improve osteogenic

differentiation (Bellows et al., 1987; McCulloch et al., 1991). These cultures stained positive for a calcified matrix and displayed bone-like phenotypes. However, it was clear that the longer in culture these cells were, the lower the potential for their osteogenic differentiation, suggesting this population of cells were progenitor cells with limited self-renewal capabilities (McCulloch et al., 1991). This was further supported by another group who were successful in isolating rat bone “marrow derived mesenchymal stem cells” (MSCs) and differentiating them into osteoblasts. Using dexamethasone and bone morphogenic protein 2 (BMP2) these cells showed high alkaline phosphatase (ALP) activity, proper gene expression and a calcified matrix. It was also shown that cells which were initially low in ALP activity could be plated again with more dexamethasone and showed increased ALP activity, suggesting isolation of even earlier progenitor cells (Rickard et al., 1994). Studies using human bone marrow derived MSCs suggested that these two different progenitor populations, one which had small colonies of cells called colony forming cells and the other with large clusters of cells called cluster forming cells, responded differently to osteogenic factors. The colony forming cells with limited proliferation represented an earlier stage of development whereas the cluster forming cells had greater proliferation representing a more mature cell (Long et al., 1995). With the understanding that bone marrow derived MSCs can differentiate into functional osteoblasts *in vitro*, the potential for use in cell-based transplantation therapies became greater. However, it had yet to be shown whether transplanted cells *in vivo* could

differentiate the same way. One study demonstrated that isolated human MSCs when injected into a mouse, either embedded in ceramic or in a diffusion chamber, were both successful in forming functional osteoblasts after being cultured in dexamethasone (Gundle et al., 1995).

Bone marrow derived MSCs however do not represent the only lineage from which osteoblasts arise. Since cranial neural crest cells (CNCs) are responsible for producing the majority of the bones in the skull, an *in vitro* approach to produce osteoblasts from CNCs helped to associate differences in etiology of craniofacial defects from other skeletal defects. The early efforts to accomplish this involved culturing mandibular and maxillary mesenchyme, derived from CNCs, from both chick and mouse and found that osteogenesis occurred when in the presence of mandibular epithelial tissues. However, it could not induce osteogenesis in non-osteogenic mesenchymal tissues or in trunk neural crest cells (Hall, 1981). This suggested that the epithelial tissues secreted specific factors that helped direct CNC derived mesenchyme to osteoblasts. After isolating pure CNC cells from the first branchial arch of developing mouse embryos, and being successfully cultured *in vitro*, one study began to develop a directed osteogenic differentiation. Using similar osteogenic supplements from mesodermal and ESC osteogenic supplements, AA, BGP and dexamethasone, they were able to confirm osteogenic differentiation through ALP activity, and specific protein expression (Ekanayake and Hall, 1997). Another lab similarly differentiated CNC cells, derived from hair follicles, into osteoblasts using vitamin D3, as well as AA, BGP, and

dexamethasone (Urano-Morisawa et al., 2017). These new methods for differentiating CNC cells into osteoblasts allowed researchers to understand molecular differences between CNC and mesoderm derived osteogenesis.

1.9.2 Pluripotent Stem Cell Differentiation

As ESC research is improving, the potential for using ESC-based therapeutics in osteogenic defects and injuries is becoming greater. ESCs are particularly attractive, specifically when considering the number of cells necessary for transplantation, when considering the potential for unlimited proliferation of ESC, whereas adult stem cells (ASCs) can only proliferate so many times before senescing. One group was successful in differentiating mouse ESCs to osteoblasts by first culturing them as EBs, and subsequently cultured in media containing different combinations of AA, BGP, and all-trans-retinoic acid. In addition, they were also able to generate osteoblasts by culturing the ESCs in a non-contact co-culture using primary embryonic osteoblasts as a feeder layer (Buttery et al., 2001). Two years later, a second study came out of our lab in which the active form of vitamin D3, 1,25αOH$_2$ vitamin D$_3$ (VD3) was used as an osteogenic inducer along with AA and BGP (zur Nieden et al., 2003). Not only was this the first used of VD3 in ESC osteogenic induction, but also did this study comprehensively analyze the timeline whereby ESCs underwent osteogenesis. The yield of osteogenesis hovered around 60% (zur Nieden et al., 2007) and was later improved to 90% by adding retinoic acid during days 3-5 of the differentiation as well as BMP-2 during late osteogenesis (zur Nieden et al., 2007).

In 2003 also, the first directed osteogenic differentiation *in vitro* from human ESCs was successful by culturing human EBs followed by supplementation of AA, BGP, and dexamethasone (Sottile et al., 2003). The zur Nieden lab later showed that VD3 was also effective in inducing osteogenesis in hESCs as evidenced by deposition of calcium (Ding et al., 2012), timely up-regulation of alkaline phosphatase activity and an appropriate sequence of expression of genes associated with osteogenesis (Sparks et al., 2018).

In vivo, hESCs can also form osteoblasts by culturing hESCs in a diffusion chamber with osteogenic supplements before transplanting into a mouse (Tremoleda et al., 2008). This gave new evidence that ESCs had the potential to be used in transplantation therapies, however, transplantation of differentiated ESCs could cause issues regarding immune rejection also known as graft versus host disease (Wu et al., 2008; Pearl et al., 2012).

1.9.3 Human induced pluripotent stem cells and osteogenesis

With the advent of mouse and human induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka., 2006; Yu et al., 2007), and their potential for patient derived cell-based therapies without a chance of immuno-rejection, it was important to show that these cells also were able to form osteoblasts. Studies from multiple groups have been successful in differentiating mouse iPSCs, as well as successful transplantation of these cells for bone repair (Li et al., 2010; Hayashi et al., 2012). One study went further to look at the differences of osteoblasts differentiated from mouse ESCs and iPSCs and found that both had osteogenic

potential as well as were expressing osteogenic genes (Ma et al., 2017). Although both cell types were generally capable of undergoing osteogenesis, the differentiation potential of the iPSCs may be limited depending on the original source of the tissue. iPSCs have been found to retain some epigenetic memory of their original cell type prior to reprogramming (Bar-nur et al., 2007). This may hinder the ability of an iPSC to differentiate into a lineage very distant in embryonic origin. Indeed, our group has shown that osteogenesis from two hiPSC lines generated in Riverside (RIV9 and RIV4) was considerably lower in calcium deposition as well as gene expression (Sparks et al., 2018). Furthermore, H9 cells differentiated through a neural crest route, whereas both RIV lines preferred to undergo osteogenesis through a mesodermal intermediate. To combat this, one group decided to generate human iPSCs from osteoblasts (hOB-iPSCs), and subsequently determine their potential to create osteoprogenitors. They found that when differentiated into EBs, these hOB-iPSCs were able to generate ectodermal and mesodermal cell types, but had difficulty forming endodermal cell types. When differentiated, these cells were able to generate cells from both the osteogenic and chondrogenic lineage, however lacked the ability to generate adipogenic cells (Roberts et al., 2017). Although, generating hOB-iPSCs may have great potential, not all institutions can generate such human tissues. To further characterize the causation of the lower osteogenic yield and differential preference of osteogenic route found in iPSCs, our study did demonstrate that it was at least partially caused by an epigenetic memory issue, specifically by a differential methylation pattern on

the *TWIST1* and *PAX7* promotors (Sparks et al., 2018). Not only did the RIV lines exhibit these faulty methylations, but other human ESC lines shared with us by collaborators also did, which suggests that although methylations were faulty in the iPSC lines, the faulty pattern may be found in bona fide hESCs as well. It remains to be answered if the faulty methylation stems from differences in the cells' culturing state and whether they may be corrected through reverting them to a naïve state.

The aims of this study are to determine whether HRTP Camel Blue effects the mitochondrial morphology of hESCS similarly to a conventional tobacco product Camel, and determine whether *in utero* exposure to HRTP Snus, causes increased susceptibility to neurological concerns such as increased anxiety and reduced motor function into adulthood.

1.10 References

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2 Chapter

Both Camel and Camel Blue tobacco smoke exposure lead to the disruption of the mitochondrial network in osteogenically differentiating human embryonic stem cells

2.1 Abstract

Previous work in our laboratory has shown that both Camel and Camel Blue, a conventional tobacco product and a harm reduction tobacco product (HRTP) lead to changes in mitochondrial function. To further demonstrate that the toxicity of these tobacco products is directly associated with the mitochondria, we employed the use of a novel and easily accessible software plugin for ImageJ called mitochondrial network analysis (MiNA) (Valente et al., 2017) to determine if the morphology of the networks is affected by both the conventional and HRTP. Exposure to both Camel and Camel Blue tobacco reduced the organization of the mitochondrial network, however Camel Blue exposure led to a greater overall disruption to the grouping, branching and area of the mitochondrial network. Addition of ascorbic acid (AA) to both Camel and Camel Blue exposed cells led to a rescue of the network morphology, while inhibition of Caspase -4 and -9 differentially rescued network morphology between the two treatments. This study highlights the benefits of using MiNA to determine changes in mitochondrial

morphology in response to toxins and demonstrates that increased risk to harm bone cells while developing is associated with the use of the HRTP Camel Blue.

2.2 Introduction

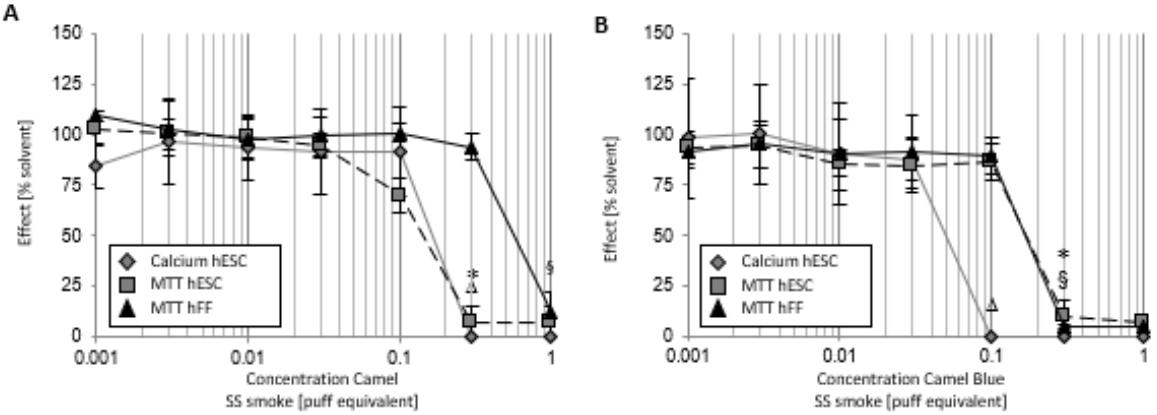
The number of diseases and health complications, which have been linked to cigarette smoking are well known to the public. Many of these health risks include damage to the airways and lungs, and the heart, leading to apoptosis and necrosis of the tissues or cancer (van der Toorn et al., 2007; Pleasance et al., 2010). However, health risks are not only limited to the smoker. Expecting mothers, who smoke during pregnancy, greatly increase the risk of the fetus having developmental defects, growth delays, and increase the chances of having the pregnancy result in a miscarriage or stillbirth (Kayemba-Kay's et al., 2010; Baba et al., 2014). With the rise of “safer” options to conventional cigarette smoking as discussed in Chapter 1, women who cannot or choose not to quit tobacco use during pregnancy, often turn to so-called harm reduction tobacco products (HRTPs).

To determine toxicological differences between conventional tobacco and HRTPs, a recent study produced by our lab utilized a human version of the embryonic stem cell test (EST). Comparing the three endpoints of cytotoxicity on human embryonic stem cells (hESCs) and human fibroblasts (hFF) and inhibition of calcification in osteogenically differentiating hESCs, we found that found that side stream smoke extract from Camel conventional cigarettes caused excessive cell death and a

measurable lack of calcification in osteogenically differentiating hESCs at 0.3 PE (Fig. 2.1A), while Mainstream smoke had no effect (Fig. 2.1C). A biostatistical model that is used to correlate half-maximal inhibitory doses determined from concentration-response curves in the three endpoints to *in vivo* embryotoxicity (Genschow et al., 2000; 2002) classified Camel sidestream smoke extract as strongly embryotoxic (Fig. 2.1C). In contrast, while cell viability was not inhibited in the range tested for Camel Blue smoke extract, Camel Blue side stream smoke ablated calcification starting at 0.1 PE, one dose lower than for the conventional Camel SS. Notably, the absence of calcification occurred in the absence of changes in cell viability up to a dose of 0.3 PE (Fig. 2.1B). While the biostatistical model also categorized Camel Blue sidestream smoke extract as strongly embryotoxic (Fig. 2.1C), it is of note that the embryotoxic effect was caused at subtoxic concentrations suggesting that this particular harm-reduction product inhibited differentiation producing developmentally toxic effects independent of cytotoxicity.

To chase after a potential mechanism of embryotoxicity, next, superoxide anion levels were determined. For Camel, there was a roughly 2.5-fold increase in superoxide anion levels, and just under a 2-fold increase in the Camel Blue exposed cells (Fig. 2.2A). Furthermore, hESCs exposed to Camel SS smoke extract exhibited significant Caspase 3/7 activation, while Camel Blue SS smoke extract-exposed cells only showed mild activation (Fig. 2.2B). The addition of ascorbic acid, an antioxidant, led to a visible decrease in Caspase 3/7 positive cells

in both cases, suggesting that ROS is aiding in the initiation of apoptosis. Indeed, following a live dead assay, a greater proportion of both injured and dead cells were counted in the cells exposed to Camel extracts, while only a minor increase in such cells was noted upon exposure to Camel Blue extracts (Fig. 2.2C).



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	IC50 hFF	IC50 H9	ID50 H9	class	categorization
Camel MS	>1	>1	>1	I	Non-embryotoxic
Camel Blue MS	>1	>1	0.51 ± 0.08	II	Weakly embryotoxic
Camel SS	0.58 ± 0.08	0.14 ± 0.03	0.17 ± 0.04	III	Strongly embryotoxic
Camel Blue SS	0.18 ± 0.03	0.16 ± 0.05	0.05 ± 0.01	III	Strongly embryotoxic

Fig. 2.1. Sidestream smoke inhibited osteogenesis and cell viability. Human ESCs were treated with different concentrations of sidestream smoke solution concurrently with osteogenesis. Cultures were assessed for calcium deposition and cell viability on day 20 using Arsenazo III and MTT assay, respectively. (A) Camel sidestream smoke solution. (B) Camel Blue sidestream smoke solution. Each graphed point is the average of three independent experiments ± standard deviation. $^{\Delta}P<0.05$ represents the lowest concentration that is significantly below the untreated control in the calcium assay, as determined by One-Way ANOVA. $^{*}P<0.05$ represents the lowest concentration significantly below the untreated control in the hESC MTT assay as determined by One-Way ANOVA. $^{\S}P<0.05$ represents the lowest concentration that is significantly below the untreated control in the hFF MTT assay as determined by One-Way ANOVA. (C) List of IC_{50} and ID_{50} values determined from concentration-response curves for both tobacco products grouped by mainstream and sidestream smoke and embryotoxicity classifications as calculated according to Genschow et al. (2000). hFF, human foreskin fibroblast; hESC, human embryonic stem cell; MS, mainstream; MTT, mitochondrial dehydrogenase activity assay; SS, sidestream. Data courtesy of Dr. Ivann Martinez and Joseph Madrid.

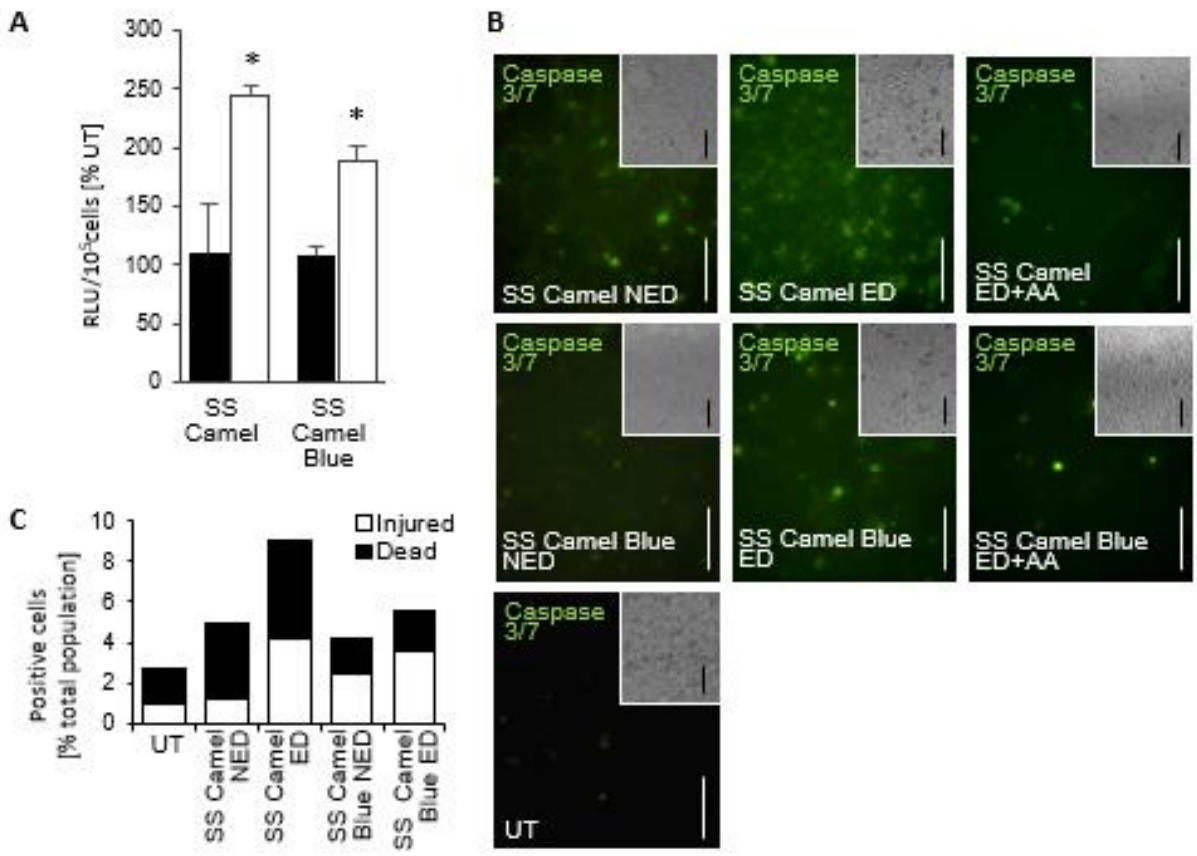

Fig. 2.2. Exposure to Camel led to a greater increase in ROS and cell death than Camel Blue. (A) hESCs exposed to sidestream Camel and Camel Blue (white bars) both cause a significant increase in the amount of superoxide anion, by a factor of 2.5, and 2 respectively to the untreated (black bars). (B) Immunocytochemistry of activated Caspase 3/7 in hESCs exposed to Camel and Camel Blue, alone and in combination to AA, shows and a greater increase in activation in Camel than Camel Blue, both however partially rescued by AA. (C) A live dead assay showed a greater proportion of cells were killed or injured due to Camel exposure, with Camel Blue having significantly fewer dead cells. *P<0.05 One-Way ANOVA versus UT Data courtesy of Dr. Nicole Sparks and Dr. Lauren Walker.

To determine the pathways in which the Camel and Camel Blue were causing cell death, RNA collected from the cells exposed to both the non-effective and effective doses of Camel and Camel Blue were probe using a qPCR apoptosis array (Fig.

2.3). A significant increase in expression of mitochondria related apoptotic genes from the BCL-2 protein family including *BCL-2, BCL-2L10, BCL-2A1 and BAX*, were found in both Camel and Camel Blue exposed cells. Additionally, a number of Caspase proteins had increased gene expression in cells exposed to Camel and Camel Blue extract. An increase in gene expression of Caspase-4 and -5 were only found in Camel Blue exposed cells, while Caspase-8 and -9 were significantly increased in both tobacco extracts, but significantly higher in Camel.

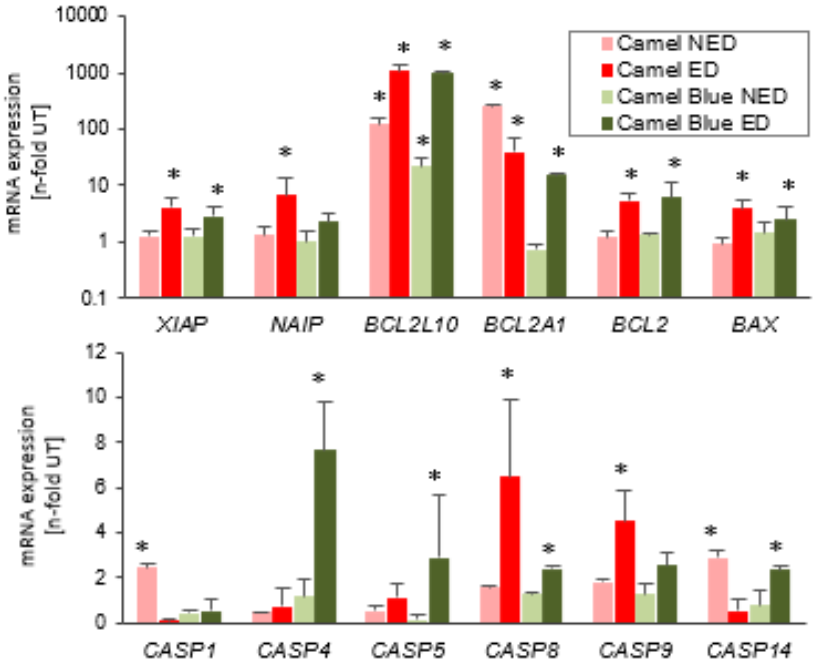

Fig. 2.3. Gene expression of hESCs exposed to both Camel and Camel Blue suggests activation of the mitochondrial mediated apoptosis pathway. mRNA expression of hESCs, exposed to both the NED and ED of Camel and Camel Blue, were increased in a number of pro-apoptosis and mitochondrial associated apoptosis genes including XIAP, BCL2L10, BCL2, BCL2A1, and BAX, while NAIP was only increased in in the Camel exposure. Additionally, expression of Caspase genes -8 and -9 were significantly increased in Camel exposed cells, and Caspase -4, -5, -8, and -4 were increased when exposed to Camel Blue. *P<0.05 One-Way ANOVA versus UT Data courtesy of Dr. Nicole Sparks.

Due to the evidence of both tobaccos causing activation of the mitochondrial apoptosis pathway, albeit at a different pace, our lab measured the mitochondrial membrane potential and ratio of AMP to ATP under Camel and Camel Blue exposure (Fig. 2.4). The membrane potential of Camel exposed cells was significantly reduced with a higher ratio of AMP to ATP suggesting a disruption of the mitochondrial membranes, an effect not noted in the Camel Blue exposed cells. Although the membrane potential and AMP/ATP ratios were not significantly altered, the evidence of gene expression changes suggests that Camel Blue exposed cells were still affected, but to a lesser extent than Camel exposed cells.

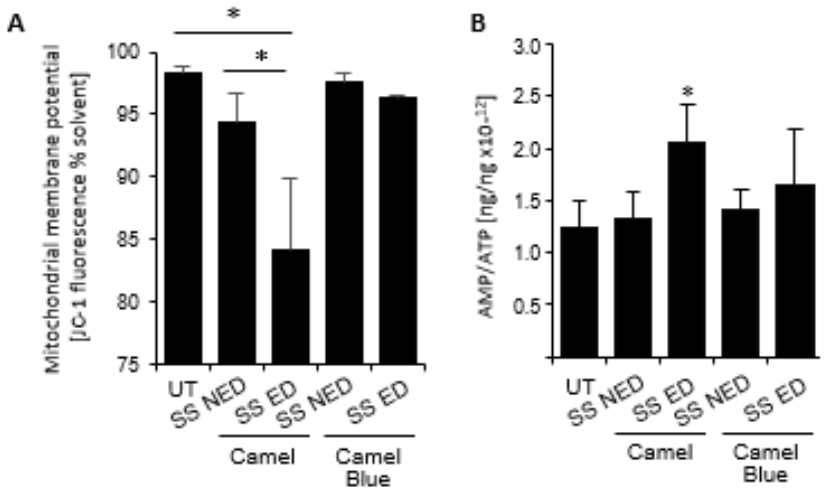

Fig. 2.4. Mitochondrial function is reduced when hESCs are exposed to Camel tobacco. (A) The mitochondrial membrane potential of hESCs was significantly reduced in the sidestream effective dose (SS ED) as compared to both the non-effective dose (SS NED) and the untreated cells, while no effect was found from Camel Blue exposure (B) A reduction in energy production, or symptoms of a low energy state was found in Camel exposed cell as shown by a higher ratio of AMP to ATP. *P<0.05 One-Way ANOVA Data courtesy of Dr. Nicole Sparks.

Increased ROS has been found to degrade mitochondrial DNA (mtDNA) (Shokolenko et al., 2009), and as a result nearby mitochondria are able to initiate mixing of mtDNA for complementation with damaged mtDNA, achieved through fission and fusion events (Yang et al., 2015). The changes in mitochondrial fission and fusion are what builds the complex mitochondrial network (Liu et al., 2009), and if the network were to be disturbed the cell is unable to regulate mitochondrial content mixing.

This is important for cell health, since mitochondria play two major roles in cell homeostasis. The first role is the production of energy through biochemical reactions such as oxidative phosphorylation in the inner mitochondrial membrane. Within the mitochondria, the tricarboxylic acid (TCA) cycle produces the electron carriers NADH and FADH2, which donate their electron to the electron transport chain (ETC). The ETC leads to the accumulation of protons in the inter-membrane of the mitochondria, which through ATP synthase, converts ADP to ATP. The final electrons are then accepted by cytochrome-c, which reduces oxygen to water via Complex-IV (Mitchell, 1961; Fernie et al., 2004). The second major role is maintaining cell homeostasis, in which the mitochondria sense cell stressors such as ER stress, oxidative stress and general toxicity, and in some cases aid in cell recovery. ER stress can occur when protein folding problems are occurring activating the unfolded protein response (UPR). Proteins on the transmembrane of the IRE1α and PERK signals to activate the mitochondrial apoptotic pathway through the BLC-2 protein family (Shore et al., 2011). As mitochondria produce

energy, they generate ROS as a byproduct, which then can negatively affect the ETC and mtDNA (Kovacic and Osuna Jr., 2005; Santos et al., 2002).

Independently of the specific toxicants, if the cell becomes over-burdened with such stress, it can activate the intrinsic apoptosis pathway, which is executed in the mitochondria. Certain toxicants are able to cause permeability to the outer membrane of the mitochondrion, which releases cytochrome-c from the inner membrane and pushes the cell into apoptosis (Garrido et al., 2006). In other cases, activation of Bax, a member of the BLC-2 family of pro-apoptotic proteins, is sufficient to cause release of cytochrome-c (Eskes et al., 1998). Once cytochrome-c is in the cytoplasm, it binds to apoptosis factor APAF-1 and facilitates the cleavage of caspases such as Caspase-9, an initiator caspase which then begins a cascade of caspase activations and other apoptosis inducing factors involved in mitochondrial mediated apoptosis (Acehan et al., 2002; McIlwain et al., 2013).

The health of the mitochondrial as well as the mitochondrial DNA (mDNA) are vital for not only general health but also for proper fetal development (Pejznochova et al., 2010). A retrospective study of 75 pediatric patients with verified mitochondrial disorders found that 45 children had some type of developmental delay and 11 which had lethal infantile mitochondrial disease (LIMD) (Skladal et al., 2003). Other fetal mitochondrial defects include mutations in the SCO2 (Cytochrome C Oxidase Assembly Protein 2) gene, which have caused congenital cardiomyopathy, encephalopathy, and neurogenic muscle atrophy leading to the

death of the fetus (Tay et al., 2004). Another study found that a woman whose family had a homoplasmic mutation within mtDNA, experienced 6 neonatal deaths, and 1 child with Leigh syndrome, mostly caused by defects in mitochondrial energy production. Autopsies from the neonatal deaths showed all suffered from mitochondrial defects (McFarland et al., 2002).

Due to potential changes in mitochondrial membrane potential, energy production, as well as damaging mtDNA, the effects of tobacco while far reaching, seem to be integrally involved with the mitochondria. In conjunction with the prior data collected in the lab, this study specifically aimed to determine whether exposure to Camel Blue sidestream smoke extract – while not causing changes to the mitochondrial membrane potential – caused other changes to mitochondrial health, which my adversely affect cell health and differentiation long-term. In this context, the usefulness of the network analysis software MiNA was used to specifically determine whether the HRTP exposure affected the mitochondria network morphology of osteogenically differentiating human embryonic stem cells. The procedures used in this study will also be applicable to other toxicological studies regarding fetal and neonatal health, and thus can be a useful tool in analyzing mitochondrial disorders.

2.3 Materials and Methods

2.3.1 Cell Culture and Treatment

H9 Human ESCs acquired from WiCell (WiCell Research Institute) were maintained in mTeSR media (Stem Cell Technologies) on Matrigel (BD Biosciences) coated plates and incubated at 37°C with 5% CO_2. H9 cells were kept undifferentiated and passaged every 3 days by dissociating cells with Accutase® (Innovative Cell Technologies, Inc.).

2.3.2 MitoTracker Staining

H9 hESCs were released from the plate using trypsinization was subsequently washed in PBS and resuspended with 200 nM MitoTracker dye (Invitrogen) and incubated at 25°C for 20 minutes in darkness. The cells were then fixed using 4% paraformaldehyde for 15 minutes at room temperature, washed three times with phosphate buffered saline (PBS), and then permeabilized with 0.1% Triton X- 100 in PBS for 15 minutes at room temperature. To counterstain the nucleus, the cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI) at room temperature for 30 minutes and washed 3 more times with PBS. The stained cells were then resuspended in PBS with 2% fetal bovine serum (FBS) and 1 mM Ethylenediaminetetraacetic acid (EDTA) and spun at 200rpm for 5 minutes onto a pre-coated Shandon Single Cytoslides (ThermoFisher) using a Shandon Cytospin 3 (Shandon) at a concentration of 5×10^5 cells/ml.

2.3.3 Image Capturing and Analysis

Using a Leica DMi8 fluorescent confocal microscope, z-stack images were captured and max projected for a flat image containing the full mitochondrial network. The flattened images were then pre-processed using the default settings of the MiNA (Mitochondrial Network Analysis) software plugin for ImageJ (Valente et al., 2017). The software then digitally created a skeletonized image of the Mitotracker DeepRed signal. MiNA then analyzed the morphology of the network, which outputs the lengths of branches and networks, the number of branches and networks, and the overall area of the network known as the mitochondrial footprint. Analysis of 10 cells was used to determine the baseline scores, while analysis of 5 cells was used per treatment group.

2.3.4 Statistical Analysis

Using IBM SPSS, a one-way anova was used to measure statistical significance. Significance was determined at a confidence of 95% or greater ($P \leq .05$)

2.4 Results

ESCs treated with Camel and Camel Blue extracts were stained with MitoTracker Deep Red, a fluorescent dye which labels mitochondria, and the mitochondrial network was imaged using confocal microscopy. The images were flattened and processed for enhancing the image, skeletonized (Fig 2.5A), and finally the network morphology was analyzed using MiNA. In the untreated cells, the mitochondrial network was more spread out and formed homogenous networks

throughout the cells (Fig 2.5B). In the effective doses of both tobacco products the networks were more congregated and denser than both in the non-effective dose as well as the untreated cells. Visibly the addition of AA did organize the general morphology of the network more similarly to the untreated cells, however network analysis further elucidated specific biometrics useful in describing the effects of tobacco exposure.

Using an open source program called Mitochondrial Network Analysis (MiNA), several endpoints are given as an output, such as number of individual non-networks, number of networks, average branch length, number of branches per network, and the area of mitochondrial footprint for example. For the MiNA software to be able to analyze the mitochondrial networks, the images captured using confocal microscopy were flattened using max projection. Due to the high magnification of the images, the mitochondrial network could appear fuzzy and distorted. Thus, to make the networks sharper and more distinct the images were processed using an unsharp mask, CLAHE, and a median filter through the ImageJ process functions. Additionally, to reduce the amount of noise in the images, a Gaussian blur was applied again using ImageJ enabling a smoother and clearer view of the mitochondrial networks. Once the images had been processed, the mitochondrial networks were skeletonized using ImageJ and these skeletonized images (Fig 2.5A) were directly processed and analyzed using the MiNA software plugin.

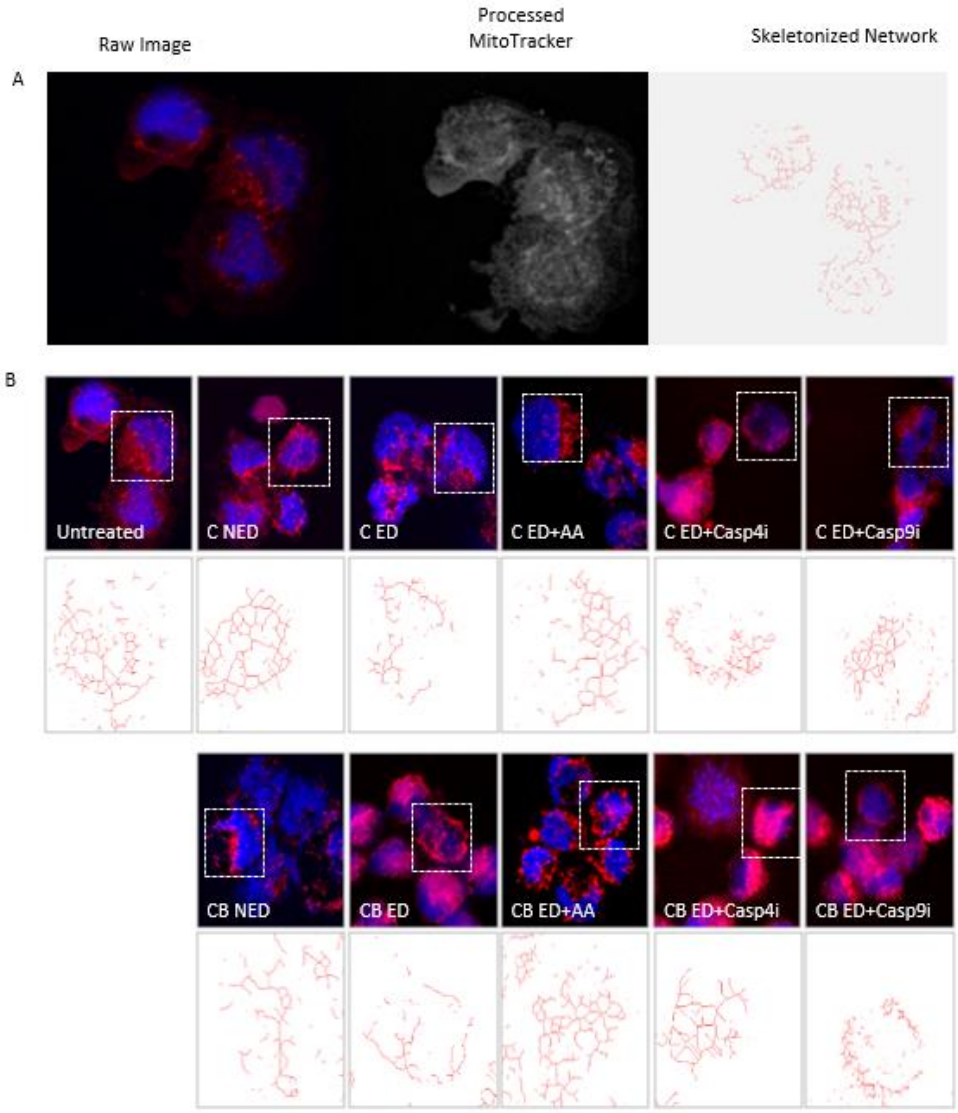

Fig 2.5 Mitochondrial network morphology is disturbed when exposed to Camel and Camel Blue tobacco extract, with rescue effect from AA. (A) Raw immunocytochemistry (ICC) image from confocal microscopy, with red staining of mitochondrion and blue DAPI of the nucleus. The MitoTracker deep red channel was processed using unsharp mask, CLAHE, median filter, and Gaussian blur to make the network sharper. Skeletonized image of the mitochondrial network as produced by Mitochondrial Network Analysis (MiNA). (B) Comparison of graphical representation of mitochondrial networks exposed to a non-effective dose Camel and Camel Blue tobacco smoke extract, and an effective dose, alone and in combination with ascorbic acid (AA), Caspase-4 inhibitor (Casp4i) or Caspase-9 inhibitor (Casp9i). The effective dose of both Camel and Camel Blue caused the mitochondrial network to become disorganized, and supplementation of AA led rescued the network morphology to appear more like the untreated cells.

The number of individual non-networks represented large round mitochondrial spots, rods, or punctate, which do not contain obvious branches. For Camel exposed hESCs the number of individuals was not significant across any of the treatments, however with Camel Blue, all the treatments including the non-effective dose had significant differences to the number of individuals against the untreated samples, except for AA (Fig 2.6A). The non-effective dose showed an increase in individuals, while the effective dose had a lower number of individuals.

A second endpoint, the number of networks, is representative of the mitochondrial networks, which contains branches. In the cells exposed to the Camel extract, the only significance found was a decrease in number of networks in cells supplemented with Caspase-4 inhibitor compared to the untreated cells (Fig 2.6B). Caspase-4 is not directly responsible to pushing the cell into apoptosis, instead, Caspase-4 has been found to cleave pro-Caspase-3 which further pushes the cell towards apoptosis (Kamada et al., 1997). For Camel Blue exposed cells, the number of networks for supplementation of the caspase inhibitors -4 and -9 led to a significant drop in the number of networks (Fig 2.6B). The non-effective dose was also significantly higher than the effective dose, however interestingly neither were significant to the untreated. Inhibition of Caspases have been shown to block cell death, however damage to the cell can still occur (Coelln et al., 2008),

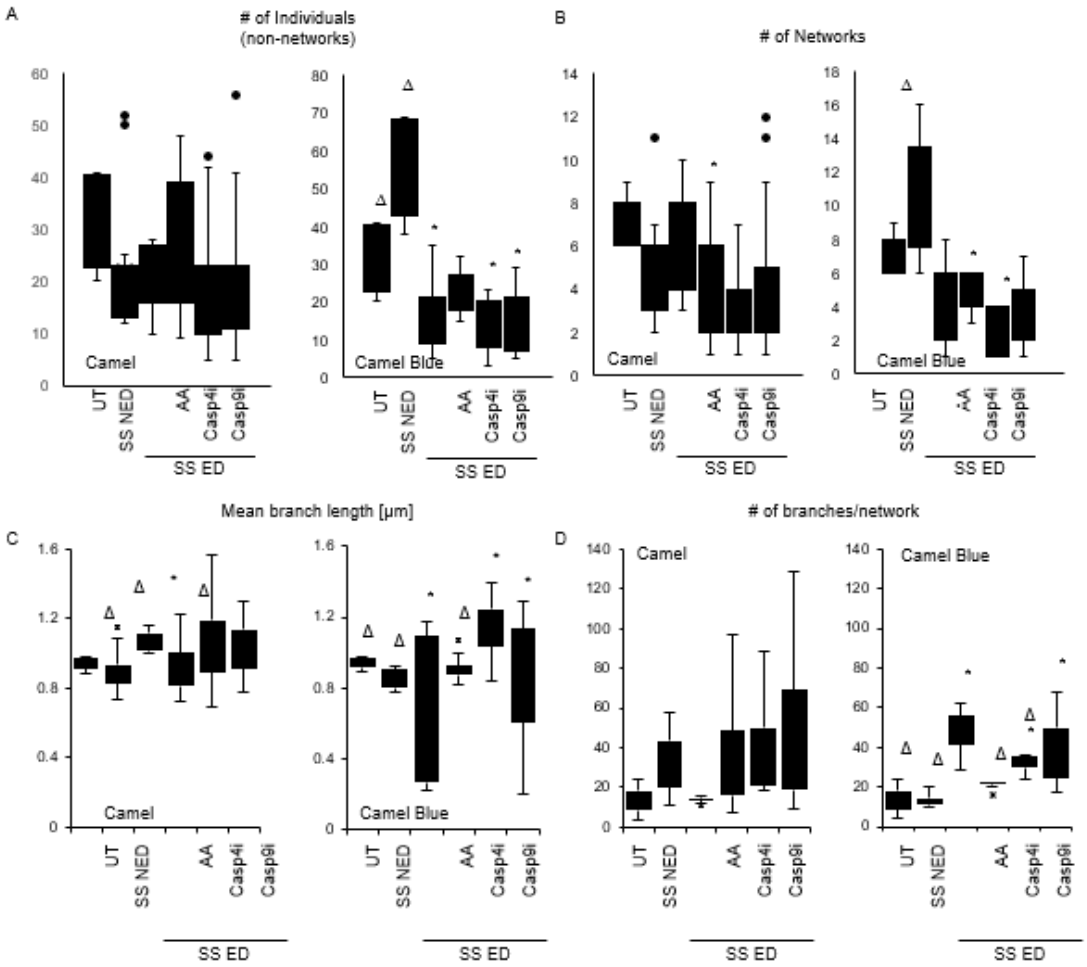

Fig 2.6 Harm reduction tobacco product Camel Blue leads to greater changes in mitochondrial grouping and branching than conventional Camel tobacco. (A) The number of non-network grouping mitochondrion had no significant changes when mESCs were exposed to the extract of Camel tobacco, however the mean number of individuals significantly increased in response to Camel Blue, with a slight rescue from ascorbic acid (AA), but not with either Caspase-4 (Casp4i) or -9 inhibitors (Casp9i). (B) Exposure to Camel again had no significant effect to the number of networks, but again a decrease was found in the sidestream effective dose (SS ED) in comparison to the SS NED from Camel Blue exposure, but no rescue effect was found. (C) The mean branch length for Camel and Camel Blue had the opposite effect where Camel resulted in an increase in length, Camel Blue exposure caused a decrease, with both being rescued by AA, as well as a rescue effect from Casp4i and 9i when added to the Camel Blue exposure. (D) The number of branches per network was also unaffected by Camel exposure, however Camel Blue caused a significant increase with a rescue effect found from AA and Casp9i. *P<0.05 One-Way ANOVA versus UT, ΔP<0.05 One-Way ANOVA versus ED.

suggesting that any rescue events from Caspase-4 and -9 inhibitors may still lead to cell damage.

Camel effective dose treated cells had a significant increase in branch length, however supplementation with AA increased the range with many cells having significantly lower lengths in a slight rescue (Fig 2.6C). Introduction of Caspase-4 inhibitor significantly increased the length and Caspace-9 inhibitor having a similar distribution to the AA treatment. Camel Blue caused a significant drop in mean branch lengths with AA supplementation being significantly different from the untreated cells, but not from the non-effective dose (Fig 2.6C). Addition of Casapce-4 inhibitor significantly increased the mean branch length along with Caspase-9 inhibitor also being significantly different from the non-effective with higher and lower lengths.

The number of branches per individual networks gives a sense of the complexity of the networks. Exposure to Camel led to no significant differences to the number of branches per network (Fig 2.6D), however Camel Blue exposure caused the number of branches per network to be significantly increased. Supplementation with AA was still increased from the non-effective dose however with a clear rescue effect (Fig 2.6D). Caspase-4 inhibitor also rescued the increased number of branches per network, although not to the same extent as AA, and Caspase-9 inhibitor was also significantly decreased from the effective dose, however with the least rescue effect.

The mitochondrial footprint in turn is representative of the total area all the active mitochondrial networks take up in the cell due to the specificity of MitoTracker Deep Red dye to only label active mitochondria. The effective dose of Camel led to a significant decrease to both the non-effective and control cells. When AA was added to cells exposed to the Camel effective dose, a rescue effect was seen significantly bringing up the total area back to similar values of the non-effective dose (Fig 2.7B). Caspase-4 inhibitor caused a further decrease in the mitochondrial footprint significantly lower than the non-effective dose. While cells treated with Caspase-9 inhibitor had a significantly lower mitochondrial footprint than the untreated cells, the rescue effect showed a significant increase from both the effective and non-effective doses of Camel exposed cells. For the Camel Blue exposed cells, a significant decrease in the mitochondrial footprint was also seen for both non-effective and effective doses, with the effective dose significantly lower than the non-effective dose (Fig 2.7B). A rescue effect was seen when AA and Caspase-4 inhibitor was added, however addition of Caspase-9 inhibitor did not significantly affect the mitochondrial footprint.

The area of the mitochondrial network takes up in the cell may be proportionate to the size of the cell. Additionally, the area of the nucleus may also show effects from the tobacco treatments, and therefore using ImageJ, the shape of the nucleus was outlined, and the area within the outline was measured. In the hESCs exposed to the Camel Blue extract a significant increase was found when

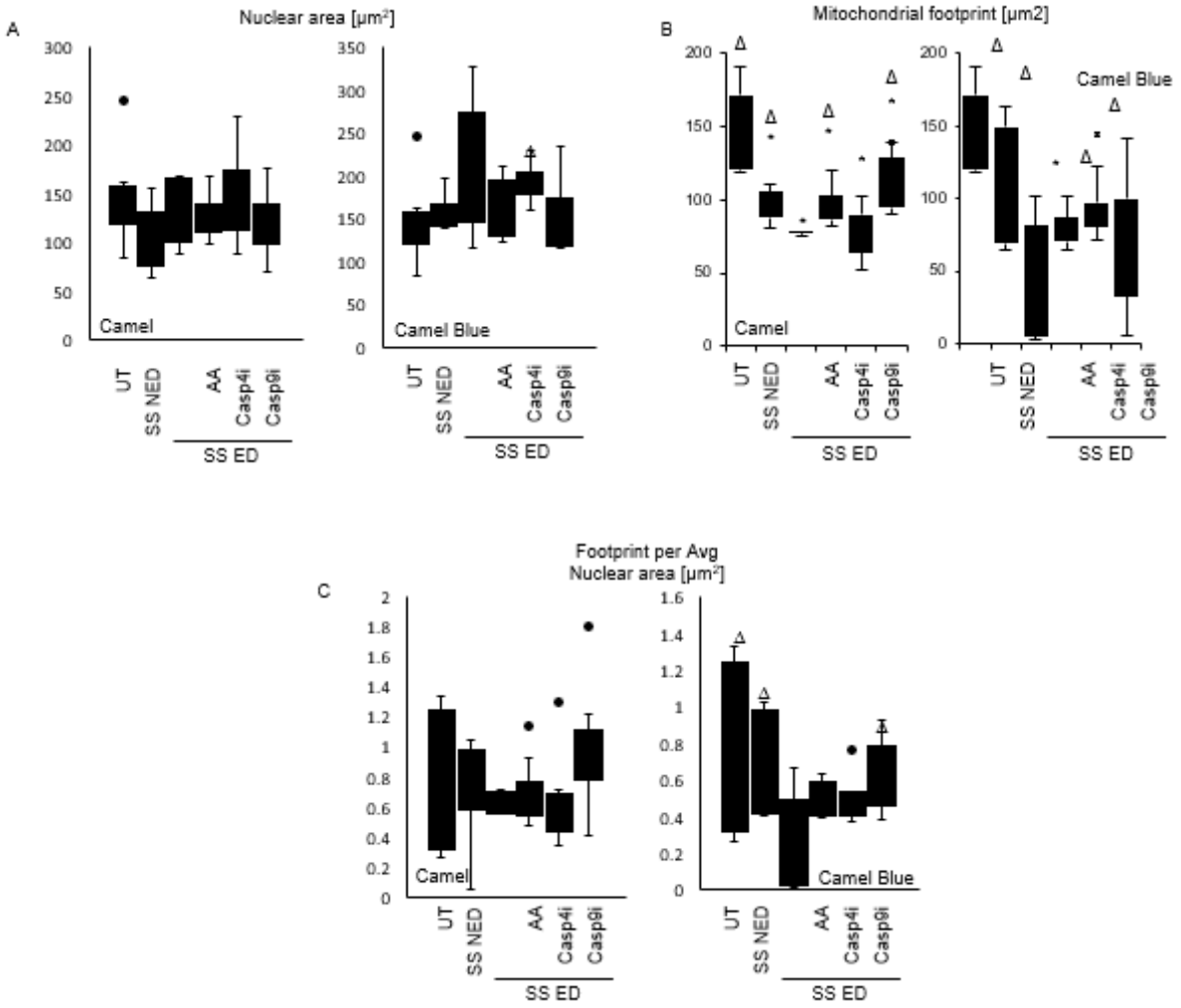

Fig 2.7 The area mitochondrion take up in the cell when normalized is only reduced in Camel Blue exposed cells. (A) Exposure of Camel to hESCs did not significantly affect the size of the nucleus, however Camel Blue exposed cells had larger nuclei. (B) The mitochondrial footprint in Camel exposed sells was significantly decreased, with a slight rescue from AA and Casp9i. Camel Blue exposed cells also had a decreased mitochondrial footprint, however with a rescue from AA and Casp4i. (C) No effect from Camel exposure was found when the mitochondrial footprint was normalized to the average size of the nucleus, however exposure to Camel Blue did significantly decrease the mitochondrial footprint when normalized, with a slight rescue effect from Casp9i only.
*P<0.05 One-Way ANOVA versus UT, ΔP<0.05 One-Way ANOVA versus ED.

comparing the effective dose to the untreated cells, with a rescue effect from both the AA and Caspase-4 inhibitor treatments, however compared to the effective dose, only the Caspase-9 inhibitor had a significant drop in the area of the nucleus (Fig 2.7A).

Comparing the mitochondrial footprint to the nuclear area helps to determine whether the significance off the footprint is lost when normalized to the nuclear area. ESCs exposed to the effective dose of Camel Blue had a significantly smaller footprint when compared to the untreated cells, and a rescue effect was again found in both the AA and Caspase-4 inhibitor treatment, but not with the Caspase-inhibitor (Fig 2.7C). Interestingly, for both Camel and Camel Blue the standard deviations of the branch length and network size across all treatments had no significance (Fig 2.8) and are therefore not good markers for tobacco toxicity.

2.5 Discussion

In many of the health issues caused by smoking, the tissue is damaged in part by responses to reactive oxidative species (ROS) produced from, and as a result of smoking tobacco (Pryor et al., 1993; Asami et al., 1996; Pignatelli et al., 2001). Increased levels of ROS have been found to alter mitochondrial functions causing the activation of intrinsic apoptosis along with a decrease in ATP production (Tiwari et al., 2002). In addition, *in vitro* evidence supports that the toxins within cigarette

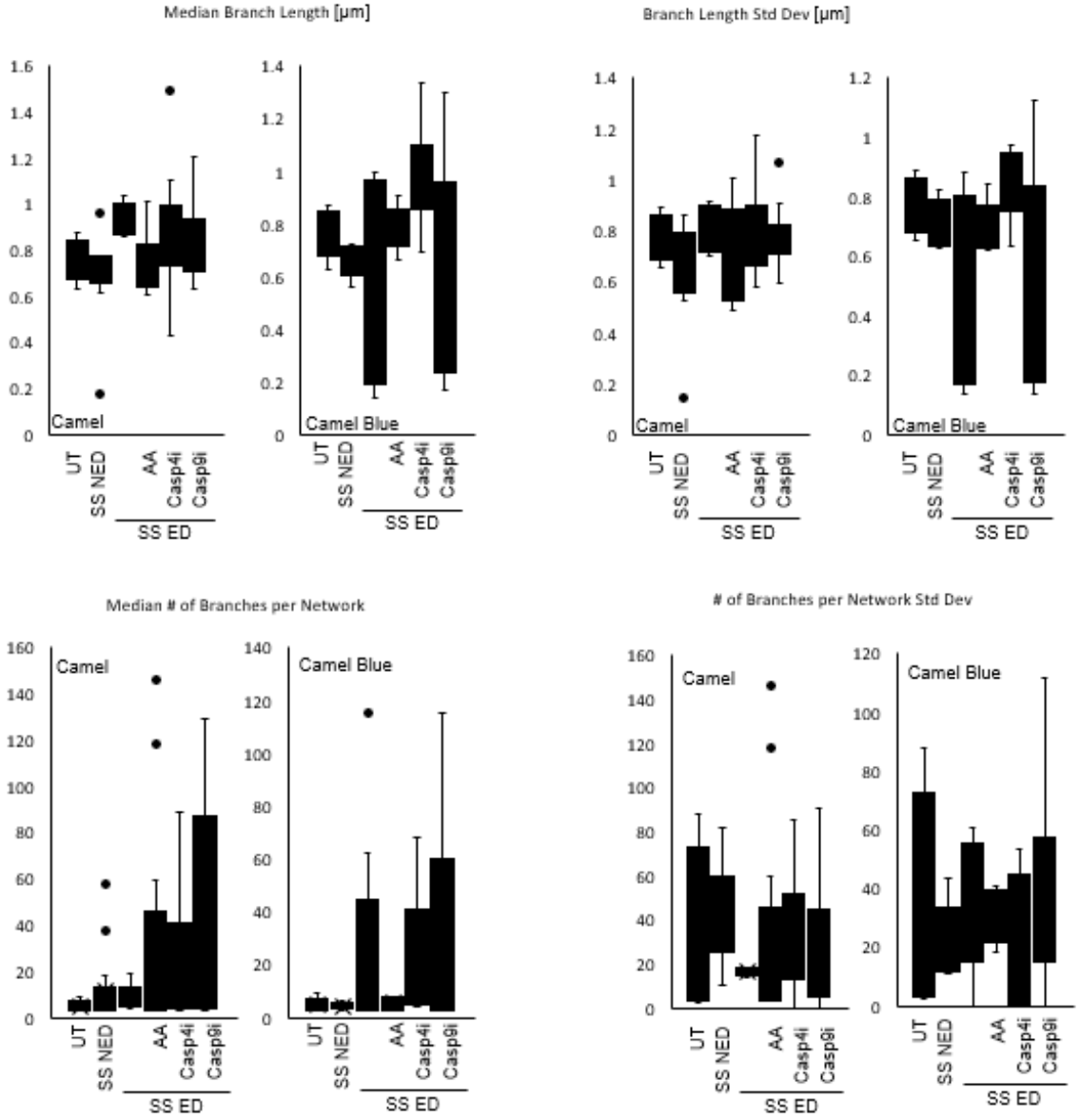

Fig 2.8 Median and standard deviation of branch lengths and number of branches per network are unaffected when exposed to tobacco extracts. Other biometric exposed cells had no significant differences between the treatments for both the nuclear are and the mitochondrial footprint normalized to the nuclear area. The MiNA software also outputs median values for branch length and number of branches per network, and measures quantified by MiNA, including the median and standard deviation of branch lengths and number of branches per network found no significant effects with exposure to either conventional Camel tobacco or harm reduction tobacco product (HRTP) Camel Blue. *P<0.05 One-Way ANOVA versus UT, ΔP<0.05 One-Way ANOVA versus ED.

smoke cause a significant increase in mitochondrial membrane potential ($\Delta\psi m$) within 3hrs exposure and with apoptosis occurring after 16hrs. Furthermore, the $\Delta\psi m$ can be rescued using certain antioxidants such as N-acetylcysteine (NAC) (Banzet et al., 1999). Smoking has also been found to alter mtDNA and function. One study found that a significant increase in COX-1 and COX-2, encoded within the mitochondrial DNA, are expressed at higher levels in in users who smoke or have smoked tobacco (Masayesva et al., 2006). Increased levels of COX-2 have been found to increase cancer growth and metastasis and the use of inhibitors of COX-1 and COX-2 have been used as anti-tumor drugs (Pairet and Engelhardt, 1996). COX-1 and COX-2 are enzymes in the conversion of arachidonate to prostaglandin H2, a type of prostaglandin which acts as a precursor for many biological processes like regulating immune function, inflammation, kidney development, reproductive biology, and gastrointestinal integrity, however overexpression of these enzymes have been found in a number of tumors (Mann and DuBois, 1999). Therefore, disruption to the mitochondrial genome, such as COX-1 and COX-2, may lead to a number of biological imbalances. Furthermore, tobacco smoke has been found to disrupt the mitochondrial membrane potential, decrease ATP production, and eventually lead to necrosis of the tissues (van der Toorn et al., 2007). Monocytes exposed to smoke from conventional tobacco cigarettes were found to have an increased mitochondrial membrane potential within 3hrs of exposure and with apoptosis occurring after 16hrs (Banzet et al., 1999). Specifically concerned with fetal health, tobacco smoke caused

mitochondria, isolated from E15 mESC fetuses, to have an altered $\Delta\psi m$ and a decrease in ATP production, which was associated with an increase in ROS within found within the cells (Naserzadeh et al., 2015). This suggests that either as a result of network morphology changes, or from tobacco specific mitochondrial dysfunction, tobacco toxicity is fundamentally associated with the mitochondria.

The health and functions of the mitochondria are linked to the morphology of the mitochondrial network through fission and fusion events (Bereiter-Hahn and Vöth 1994; Liu et al., 2009; Chan, 2012), and therefore determining how certain compounds affect network morphology may give insight to the mechanisms of toxicity. The MiNA software is a free open source tool that enables a quick and broad analysis to changes in network morphology. To use MiNA, the images do not need to be from an expensive confocal microscope either, they can be analyzed by any type of fluorescent or radio-labeled images, enabling smaller labs to be able to use this powerful tool. However, due to the need of a 2D image for processing, the actual 3D nature of these networks is lost and may not provide an accurate representation of certain areas within the network. Furthermore, the image quality plays a major role in determining the accuracy of the analysis. Poor quality images, or images with low resolution do not appear as crisp and the skeletonized images may not give a good representation of the network morphology. A new ImageJ plugin was created this year by Ahsen Chaudhry called Mitochondria Analyzer, which is not only capable of analyzing network morphology from 2D images, but also 3D and 2D and 3D time-lapse videos

(Chaudhry, 2019). Another group has also successfully reconstructed and analyzed mitochondrial networks in 3D using extremely high-resolution images captured using electron microscopy (Vincent et al., 2019). The use of 3D imaging and computer modeling allows for precise quantifications of network morphology greater than what can be measured using 2D images.

Using the MiNA software, the number of individual mitochondrial groups are measured separately from networks containing branches. A study by Vladimir Skulachev found that the long filamentous and branched networks of mitochondria "can represent electrically united system" which may enhance the mitochondria's ability to detoxify the cell and increase energy production (Skulachev, 2001). Therefore, it is suggested that cells which have mitochondrial networks with fewer individuals may be healthier or in a higher state of activity. While the number of individuals for Camel exposed cells was not significant across any of the treatments, Camel Blue exposed cells shared the same trend of significance for both numbers of individuals and networks. Exposure of the effective dose of Camel Blue decreased both the number of individuals and networks suggesting that an overall loss of mitochondria was occurring. Interestingly, the AA and Caspase-4 and -9 inhibitors had no rescue effect, which shows that neither ROS nor apoptosis was specifically causing this loss of mitochondria.

Decreases in the mitochondrial branch length of fibroblasts from patients with Sporadic Alzheimer's Disease have been attributed to delays in the recovery of mitochondrial networks in (Martin-Maestro et al., 2017). Furthermore, in microglial

cells addition of lipopolysaccharide (LPS) activates the microglia similar to inflammatory signals. During this activation the lengths of the mitochondrial branches decrease until returning to normal after 12hrs, suggesting that changes to branch lengths can be transient. The branch lengths of Camel and Camel Blue had the opposite effect from each other. In Camel, branch lengths increased, while in the HRTP Camel Blue the branch lengths generally showed a greater decrease. However, for both tobacco products the addition of AA lead to a rescue of the effect from the tobacco. Mitochondria are known to easily uptake and process AA (Li et al., 2001), which has been found to inhibit apoptosis by scavenging ROS (Wenzel et al., 2004). The rescue effect of the branch lengths from the addition of AA affirms the previous studies showing that AA can aid the cell during high levels of ROS from tobacco.

Inhibition of Caspase-4 and-9 in the Camel did not lead to significant differences, however the range of data is much wider than the other treatments. With Camel Blue, the addition of Caspase-4 and -9 both lead to a rescue, however Caspase-4 and -9 overshot and had longer branch lengths than the untreated, however the rescue from Caspase-9 exposed cells was not as robust as Caspase-4. Perhaps the blockage of apoptosis allowed enough time for the mitochondrial network to correct itself, but due to an over accumulation of ROS the mitochondrial network was in constant repair mode.

While Camel did not significantly alter the branches per network, addition of AA, and both Caspase inhibitors seemed to slightly increase branching, however not

significant. In Camel Blue exposed cells the ratio of branches per network was significantly altered in the effective dose and rescued by all treatments, with AA having the greatest effect and Caspase-9 inhibition with the least rescue effect. Similar to branch lengths, the motility and changes to the network when under stress is expected, however if the problem affecting the mitochondria is due to high ROS then AA should be able to take burden of the cell allowing the mitochondrial network to come back to its baseline. Exposure of Camel tobacco to hESCs caused a significant decrease in the number of mitochondria as found by the decrease in the mitochondrial footprint. However, addition of AA and the Caspase-9 inhibitor both rescued the effect back to the untreated levels suggesting that the both the inhibition of ROS and apoptosis may allow the cell enough time to fight any major losses in active mitochondria. The same effect was found with the Camel Blue treated cells, with a rescue from AA, but this time the Caspase-9 inhibitor was unable to rescue the drop in the mitochondrial footprint. Interestingly, these results suggest that both Camel and Camel Blue decrease the number of mitochondria in a cell, however from the numbers of individuals and networks was not decreased in the Camel exposed cells. Perhaps this may have to do with the density of the groups of mitochondria, a denser network may appear as having a smaller mitochondrial footprint using the MiNA method of analysis.

The mechanisms which control the size of the nucleus are still being explored, however pathologists are using the increases in nuclear size do stage the progression of certain cancers in part due to changes in the nuclear laminin

proteins (Chow et al., 2012; Nandakumar et al., 2012). While Camel extract exposed cells had no significant changes to nuclear size the Camel Blue effective dose had a significantly larger nucleus compared to the untreated. However, the nuclear area was also used to normalize the mitochondrial footprint to ensure that it was not changing proportional to the general size of the nucleus or cell. Although the mitochondrial footprint was significantly decreased in the Camel exposed cells, when normalized to the average nuclear area of cells within the treatment groups showed no significant differences. However, in the Camel Blue exposed cells, we saw that the decrease in mitochondrial footprint was not influenced by the size of the nucleus and was therefore a significant effect of exposure to Camel Blue.

2.6 Conclusion

This study uses a novel approach to analyze the network morphology of mitochondria and was found to be a useful tool which can also be applicable for understanding the effects toxins, drugs, or genetic manipulations have on network morphology. The goal of this study was to determine the morphological effects of exposure to conventional and harm reduction tobacco products on the mitochondrial networks of cells undergoing differentiation into osteoblasts. As mentioned in Chapter 1, the stigma of HRTPs being less dangerous poses a threat to women who smoke and are or are planning on having children. The relationships between HRTPs and fetal health are just now starting to be investigated, and the use of hESCs in this study directed the focus on fetal health.

Overall, the use of either conventional tobacco or HRTPs led to changes in network morphology compared to the untreated cells and or the non-effective doses, and neither product appeared significantly more harmful than the other. Furthermore, the use of AA led to the greatest rescue effect on the network morphology in all three measurements. The rescue effects by blocking apoptosis with Caspase inhibitors was always less than the effect from AA. This is likely due to the way these two different treatments work. A study which found that the inhibition of caspase-3, -6, and -9 in apoptosis induced dopaminergic cells prevented the death of the cell, however leaving the cell further damaged (Coelln et al., 2008). The addition of AA allowed the mitochondria to fight off the damaging effects from ROS, which if untreated would push the cell into intrinsic apoptosis leading to the activation of Caspases. By only inhibiting apoptosis and not treating the problem directly, the cell is unable to fully rescue itself. It would be useful to know whether the addition of both AA and Caspase inhibitors lead to a greater rescue than individually. The data acquired from MiNA followed expectations set from previous data suggesting defects in the mitochondria, whereby function is dependent on form. The ease of access and streamlined protocol for image analysis of mitochondrial network using the MiNA software enabled us to quantify hundreds of branches and networks at a much higher rate than could be done manually and proved a useful tool in analyzing the network mitochondrial network morphology. Ultimately, while Camel Blue exposure did not damage the cells such that apoptosis was fully executed, their mitochondrial health was negatively affected by

the exposure, which may be tied to the noted adverse effects in differentiation behavior.

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3 Chapter

In utero exposure to Snus leads to sex-specific anxiety-like behavior and diminished motor control function in adult mice

3.1 Abstract

The consumption of Snus, a form a tobacco claiming a reduced harm, and other non-combustible tobacco products has increased in popularity over smoking conventional cigarettes. Harm reduction tobacco products (HRTPs) are an

attractive option to women, who are unable to quit smoking during pregnancy in hopes to reduce the risk to the developing fetus. To determine whether *in utero* exposure to Snus affects anxiety and motor control function, pregnant female mice were exposed to a Snus extract via tail vein injection and pups were allowed to reach adulthood. At the age of 14 months, *in utero*-exposed mice were analyzed for behavioral and motor control function using the elevated plus maze (EPM) and the Suok Test. Analysis revealed that female mice, exposed *in utero* to Snus, were more likely to experience increased anxiety and reduced motor functions than male mice compared to a saline injection. These results suggest that Snus may not be a safe alternative during pregnancy, and that mothers, who use Snus during pregnancy, may have an increased risk of having their children be more susceptible to anxiety-like behavior and diminished motor control function.

3.2 Introduction

The adverse health outcomes associated with smoking conventional cigarettes, such as diseases of the lungs, heart, throat, and mouth, as well as cigarettes being carcinogenic (Center for Disease Control and Prevention, 2010), have been studied for almost 70 years. Many of these studies regarding toxicology of tobacco and tobacco products focus on the physical health of the subject or, in the context of developmental toxicology, their offspring. However, psychologists and behavioral scientists have questioned the effect tobacco products have on mental health and behavior. Advancements in techniques and the use of animal model

validation studies have allowed scientists to more accurately measure and analyze animal behavior in hopes of similarities to human models. There is a wide range of behavioral tests which can measure anxiety, memory, social interactions, and hierarchy structure, but some of these tests are able to measure secondary traits such as motor control.

Using a behavioral test known as the EPM (elevated plus maze), researchers can determine anxiolytic and anxiogenic behaviors of small rodents. Injection of nicotine into adult male mice initially led to an anxiogenic, causing the mice to spend less time in the open portion of the maze (Elliott et al., 2004). However, a tolerance was found to build within the mice, and the effects of the nicotine became anxiolytic, resulting in the mice spending more time in the open arms of the maze (Biala and Budzynska, 2006). The effects of nicotine on anxiety has been found to affect females differently than males and this sexual difference has been shown in both rodent and human studies (McCarthy et al., 2018; Yochum et al., 2014; Caldarone et al., 2008; Brennan et al., 1999; Fergusson et al., 1998; Orlebeke et al., 1999). One study using the EPM, found that female mice which were chronically exposed to nicotine in their drinking water, showed less time spent in the open arms when compared to male mice. A sexual difference was also found in locomotor activity, where high doses of nicotine increase motor activity in both male and female mice, however, at a lower dose, males were more susceptible to nicotine's effect on locomotor activity (Caldrone et al., 2008).

However, nicotine is not the only chemical in cigarettes; of which there are an estimated 7,000 different chemicals found or produced by the action of smoking (Talhout et al., 2011). During gestation in mice, exposure to tobacco smoke caused male offspring to show increased locomotor activity and number of times self-groomed than female mice, in addition to increased aggression (Yochum et al., 2014). Another study however, has shown that tobacco exposure during gestation led to a decrease in locomotor activity in both male and female mice during infancy and adolescence, but consisted throughout adulthood in males only. Additionally, prenatal tobacco exposed mice showed a lower distance traveled in the open arm of a maze, suggesting an anxiogenic effect (Torres et al., 2019).

Based on a vast amount of research including the studies discussed above, the public knowledge of smoking-associated health risks has improved and led to a decline in the use of conventional cigarettes within recent years. In contrast, the production of harm-reduction tobacco products (HRTPs) has led to an increase in usage by teens and women who are or may become pregnant (Soneji et al., 2017; Zhu et al., 2013). HRTPs are products which have been previously called “light” cigarettes or non-combustible products and include chewing tobacco and tobacco packets. Due to HRTPs being newer to consumers and marketed as safer, HRTPs must be studied carefully to determine negative impacts on health in contrast to conventional cigarettes.

One of the newer HRTPs vigorously marketed across North America is Snus, a popular form of HRTPs, which is a ground moist tobacco placed orally between

the gums and cheek, either loose or in a small pouch. Originating in Sweden, this product has become widely used as an alternative option to smoking conventional cigarettes. A study from 2002 found that in Sweden, 23% of men and 4% of women used Snus, whereas 15% of men and 20% of women smoked conventional tobacco products (Roth et al., 2005). An American study done in 2012 with over 10,000 people surveyed, either current, former or non-smokers, unveiled that over 20% of men and roughly 8% of women have used or currently use Snus. The study also found that 24% of 315 Snus only users, chose Snus because it was easy to use when they could not smoke, and 26% used Snus to quit smoking (Zhu et al., 2013).

While Snus is a non-combustible form of tobacco, there are still increased risks associated with cancer causing carcinogens. The debate whether Snus use leads to increased risk of cancer is ongoing, and while some studies have shown that there is no major increased risk to oral, lung or pancreatic cancer (Lee, 2013; Roth et al., 2005), other studies have shown a positive correlation to Snus use and cancer (Roosaar et al., 2008; Boffetta et al., 2005; Janbaz et al., 2014). Although, these differences in cancer rates may also have to do with the population that is being studied. Positive correlations between Snus use or other smokeless tobacco products and an increased risk of cancer have been found in the U.S., Asia, India, and Sudan, however no increased risk has been found in Northern European countries, suggesting an additional environmental factor may affect cancer rates (Boffetta et al., 2008).

Snus has been found to contain nicotine and tobacco-specific n-nitrosamines (TSNAs) including N'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Stepanov et al., 2012; Lawler et al., 2013). And while levels of TSNAs are lower in Snus than in conventional tobacco products (Österdahl et al., 2004; Stepanov et al., 2008), NNN and NNK are well known carcinogens (Hoffmann and Djordjevic, 1997; Gupta et al., 1996). Recently, a study published from our lab has found that exposure to NNN decreased osteogenic differentiation of hESCs in the absence of cell death (Martinez et al., 2019). This suggests that abuse of tobacco products containing TSNAs such as NNN, may increase the risk of perturbing bone healing and remodeling, as well as skeletal development in the womb.

Being a major contributor to the recent growth of the tobacco industry and its newer emergence to the market, the adverse health outcomes of Snus exposure, with regard to behavior, have not been sufficiently explored. Therefore, Snus exposure needs to be compared to conventional cigarette exposure to make appropriate claims about their safety. Thus, this study aimed at determining the changes in behavior of adult mice that were prenatally exposed to smokeless tobacco extract from Camel Snus while developing *in utero*, data that does not exist to date.

3.3 Materials and Methods

3.3.1 Snus Extraction

The Snus extract was prepared in a 10% (w/v) stock using 10 grams of Camel Snus in 85 mL of sterile PBS supplemented with 15% FBS, and incubated overnight at 4°C. After overnight incubation, the solution was centrifuged at 450 × g for 10 minutes at room temperature followed by another centrifugation at 13,000 × g for 1 hour. The collected supernatant was adjusted to a pH of 7.4 and brought back to the initial volume of 100 mL using PBS supplemented with 15% FBS.

3.3.2 Animals Housing and Exposure

In accordance with the guidelines for care and use of laboratory animals and approved by IACUC (Institutional Animal Care and Use Committee) at the University of California, Riverside (AUP#20180064), 10 breeding pairs of mice for each treatment group, with a mixed background of 129/Sv and C57BL/6 purchased from Charles River Laboratories, were timed bred and separated the following morning. The pregnant dams were housed individually with a 12-hour light-dark cycle at of 23 ± 2°C with a relative humidity of 50 ± 10%, and constant access to food and water. 10 pregnant dams were injected with 100 µl of 10% Camel Snus extract and 10 pregnant dams with PBS on days E6.5 and E8.5 of pregnancy. The pups from both treatment groups were housed under the same conditions and

allowed to age to 14 months prior to participation in the behavioral tests. Mice were allowed to acclimate to the testing room for 30 minutes prior to the tests.

3.3.3 Elevated Plus Maze

The elevated plus maze was made using PVC tubing as the base, and black acrylic as the floors and walls of the arms. The four arms, 50 cm long and 10 cm wide, sat 50 cm above the floor and were made up of 2 open arms and 2 walled arms, which are 30 cm high, designed to match the EPM by Padovan and Guimarães (Padovan and Guimarães, 2000). The lighting of the maze was controlled with the open arms being 30 lux and the closed arms being 3 lux. The mice were placed in the center of the four arms and allowed to explore for 5 minutes. Video recording of the exploration was used in conjunction with BORIS (Behavioral Observation Research Interactive Software) to record the number of entries into each arm, the time spent within each arm, as well as the number of times the mouse looked down.

3.3.4 Suok Test

Modeled after the Suok Test by Kalueff and Tuohimaa (Kalueff and Tuohimaa, 2005), a 2.6-meter aluminum tube with a 2 cm diameter was suspended 20 cm above the floor using two stands made of 1 cm thick clear plexiglass (50 cm x 50 cm), used to wall off the ends of the pole (Fig. 3.1). The middle of the pole was marked, and 10 segments, 10 cm long, were marked on either side of the middle.

The mouse was placed in the middle and allowed to explore for 5 minutes. Video recording in addition to BORIS was used to analyze the behavior.

Fig. 3.1. Rod built for the Suok Test**.**

3.3.5 Statistics

Using Statistical Package for Social Sciences (SPSS) software from International Business Machines (IBM), one-way ANOVA (analysis of variance) as well as the students T-test were used to determine significant changes to the mean. Additionally, significance of distribution, median and range was determined using the non-parametric Wald-Wolfowitz Runs Test, the Median Test, and the Moses Test of Extreme Reaction, respectively. Significance was determined at a confidence of 95% or greater ($P \leq 0.05$).

3.4 Results

3.4.1 Elevated Plus Maze

Comparing the effect of Snus extract to control mice, the distribution of total percent of time spent in the open arms by female mice was significantly greater in the Snus extract exposed mice, whereby the control mice had an upper and lower

value of 24% and 2.69% respectively, to 46% and 0% time spent in the open arms. No difference in percent of time spent in open arms was found within the male population. As expected, this result was also found when comparing the total amount of time spent in the open arms by Snus extract-exposed females, whereby control mice had an upper and lower value of 76.6 and 8.5 seconds, respectively, and Snus extract exposed mice had an upper and lower value of 136.7 and 0 seconds spent in the open arms (Fig 3.2A). A measure of anxiety, known as the anxiety index, was calculated from the duration of time spent in the open arms in relation to the number of times the mouse entered the open, middle, or closed sections of the elevated plus maze. Again, only female mice had a significant change in the distribution of the anxiety index score in the Snus treated mice. PBS-exposed mice had an upper and lower anxiety index value of 0.91 and 0.72, respectively, while Snus extract-exposed mice had an upper and lower value of 0.39, suggesting a sexual difference in the effect tobacco exposure has on anxiety (Fig 3.2B). No significant effects were found when analyzing the number of times in each arm, the latency to enter the first arm, or in the total number of actions made by the mice.

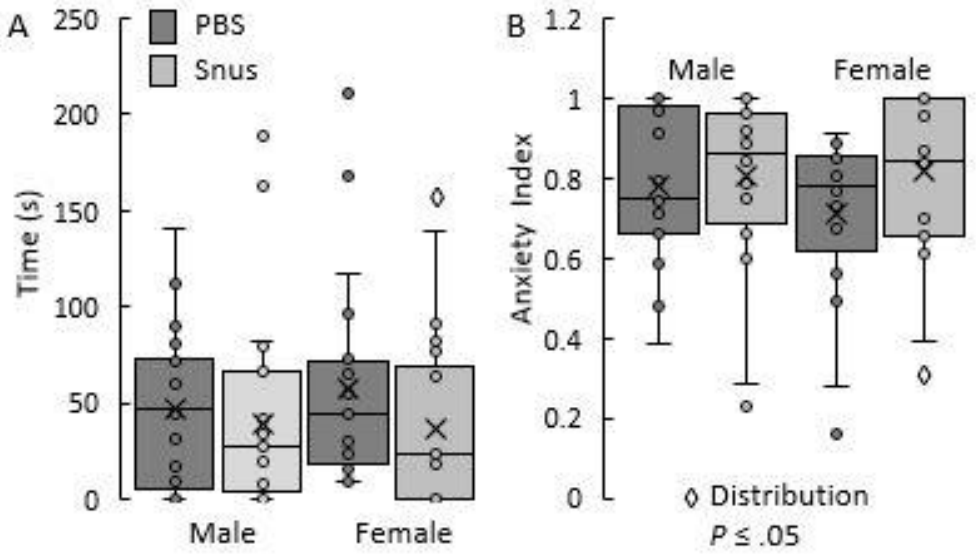

Fig. 3.2. Anxiety caused by *in utero* Snus exposure leads to sex specific differences. (A) Snus exposed male and female mice spend the same average amount of time exploring the open arms of the elevated plus maze. (B) Male and female snus exposed mice show no differences in the average of their anxiety index. However, the distribution of the data from Snus exposed females was significantly different from their control. *P<0.05 Wald-Wolfowitz Runs Test versus PBS.

3.4.2 Suok Test

Several data points can be taken during the Suok test to measure anxiety and motor control. Head dips, when the mouse looks downward towards the floor, were significantly increased in the Snus extract-exposed mice, however, when separated by sex, neither the male or female population had a significant difference in the mean (Fig 3.3A). However, Snus extract exposed males had significant changes in the range of head dips compared to the control mice.

When comparing the number of missteps, Snus extract-exposed mice had significant changes to the mean, median, range, and distribution when compared to the control mice. However, when taking sex into account, all the significant changes were attributed only to the female mice, with no significant changes to males (Fig. 3.3B), again demonstrating sexual variation in males and females in response to Snus extract.

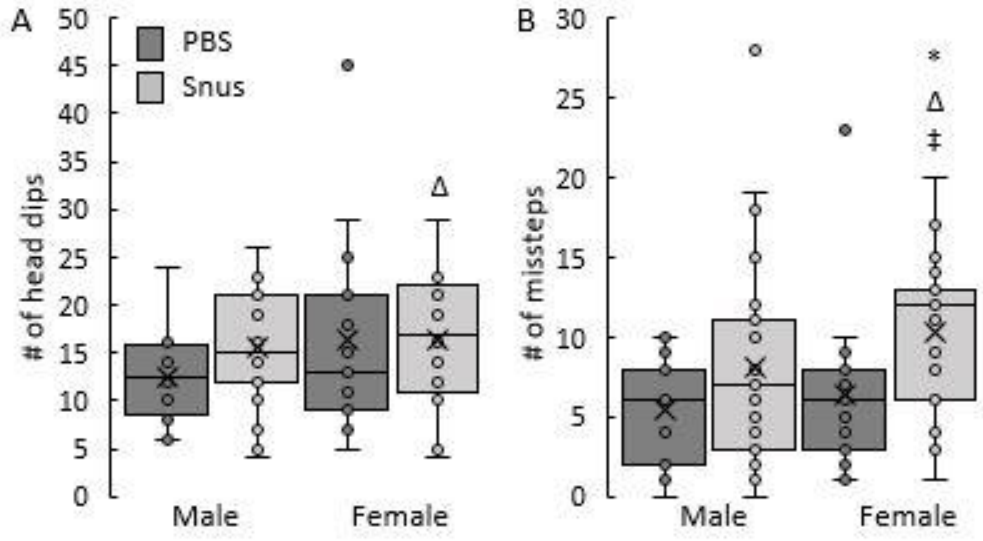

Fig. 3.3. Female mice have increased activity with reduced motor control as a result of *in utero* Snus exposure. (A) Mice exposed to Snus *in utero* had no significant differences in the mean number of head dips during the elevated plus maze, however the number of head dips for females had a significantly different range of data. (B) Female Snus exposed mice had significantly more missteps within 5 minutes than males, as well as a significant difference in the range and median of their scores. $P<0.05$ * One-Way ANOVA, Δ Moses Test of Extreme Reaction, ‡ Median Test versus PBS

Defecation and urination are measures of anxiety or fear, and while no significant differences were found for number of times defecated by males, Snus extract-exposed females showed a significant difference in the distribution of times

defecated in comparison to PBS-exposed mice (Fig 3.4A). However, the range of values for number of times urinated was significantly different for both male and female Snus extract-exposed mice in comparison to their respective PBS-exposed mice (Fig 3.4B). This result was also found for the number of times the mice groomed themselves. Both male and female Snus extract-exposed mice showed a significant change the range of the number of times they groomed themselves (Fig 3.4C), suggesting that this behavioral effect was shared between sexes. The latency to leave the center of the bar had a significant change to the distribution in female Snus treated mice with an upper value of 183.4 and 6.02 seconds respectively (Fig 3.4D), as well as a significant change in the range of the number of times the mouse fell. Of note, this effect seemed to be due to a few mice with high rates of falls not seen in the PBS-exposed mice. Next, as a measurement of motor control, the number of missteps were measured. Snus extract-exposed mice had significant changes to the mean, median, range, and distribution when compared to PBS-exposed mice. However, when taking sex into account, all the significant changes were attributed only to the female mice, with no significant changes to males (Fig 3.5A), again demonstrating sexual variation in males and females in response to Snus extract.

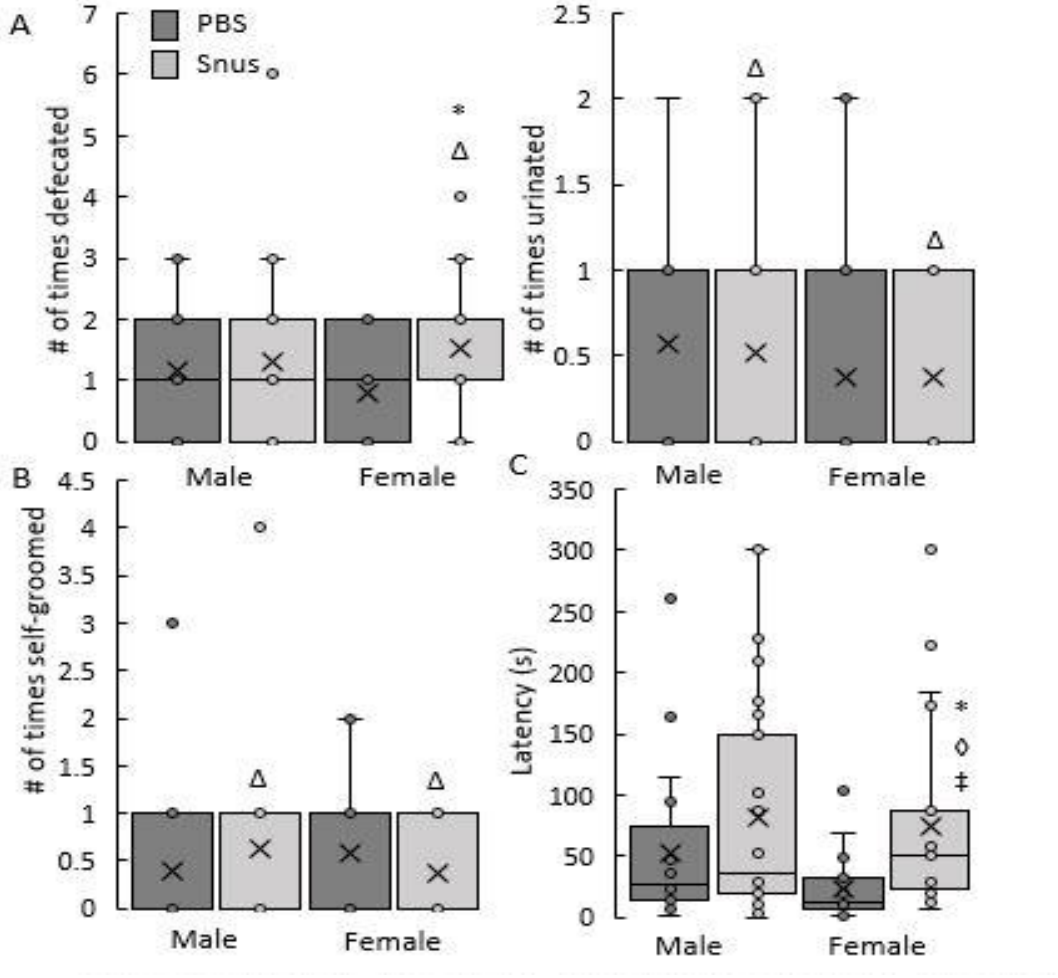

Fig. 3.4. Female mice exposed to Snus *in utero* leads to increased symptoms of anxiety. (A) Snus exposed females defecated significantly more during the Suok test than the control mice. However, both male and female Snus exposed mice had significant changes to the range of number of times urinated. (B) The range of data for number of times self-groomed for males was significantly shifted higher, while females had a significant reduction in the range. (C) The latency for Snus exposed females was significantly longer than control females, in addition to a significance in the range and median of their scores. $P<0.05$ * One-Way ANOVA, ∆ Moses Test of Extreme Reaction, ◊ Wald-Wolfowitz Runs Test, ‡ Median Test versus PBS

Additionally, we added another endpoint by analyzing the distance traveled compared to the number of missteps taken, representing the distance traveled before a misstep occurred. Snus extract-exposed mice showed a significant decrease in the distance traveled before having a misstep. However, when taking sex into account, while both male and female mice exposed to Snus extract showed a significant difference in the median values of distance per missteps, only males showed a significant decrease in the mean (Fig 3.5A).

While missteps alone, and in comparison to distance traveled, have been previously used to measure motor control (Santiago and Huffman, 2014), we have additionally developed a novel way of measuring motor control: the coordination index. This novel index takes into account head dips, side looks, distance, missteps and falls, all of which involve balance and coordination. Male mice had no significant changes in the coordination index between the Snus extract-exposed and PBS-exposed mice. In females however, there was a significant decrease in the coordination index of Snus treated mice, as well as significant differences in the distribution of scores in comparison to the PBS-exposed mice (Fig. 3.5B). Interestingly, PBS-exposed female mice had a significantly higher coordination index when compared to PBS-exposed males.

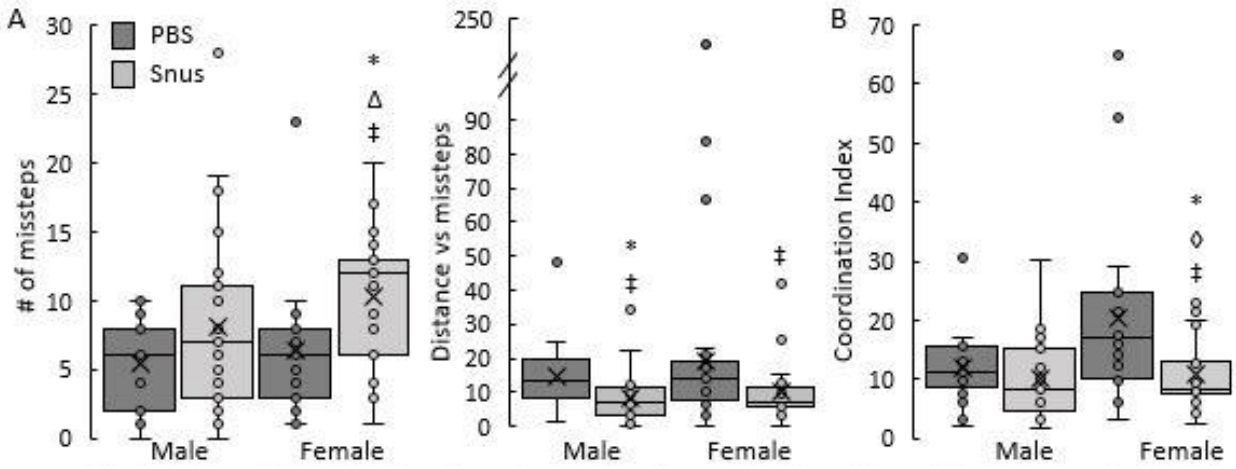

Fig. 3.5 Male and female mice show signs of motor control functions after exposure to Snus *in utero*. (A) In the full duration of the test, female mice exposed to Snus showed to have an overall higher number of missteps in the Suok Test, however when the distance traveled was factored in, only male mice had a significant change in the average distance before a misstep occurred. (B) A novel analysis called the coordination index showed a significant loss of overall coordination in female Snus exposed mice, but not males. P<0.05 * One-Way ANOVA, ∆ Moses Test of Extreme Reaction, ◊ Wald-Wolfowitz Runs Test, ‡ Median Test versus PBS.

3.5 Discussion

The marketing of tobacco products as being less harmful to conventional tobacco products may specifically appeal to women who are pregnant or are trying to become pregnant and are having difficulties with smoking cessation. HRTPs, which are said to contain either less nicotine, tar, or other additives in addition to either having a longer filter or are non-combustible, have potential dangers to the unborn child, which could lead to many health concerns, whether physical or mental. To determine potential defects behavioral and motor control function of mice exposed to Snus, a specific type of previously untested HRTP, here, we

injected pregnant dams with smokeless tobacco extract made from Camel Snus or PBS and examined the behavior and motor functions of the pups once they reached 14 months of age.

For decades researchers have been describing different behavioral problems associated with the children of mothers who smoked during pregnancy. A study from the Netherlands found that out of 1,377 twin pairs between the ages of 2 and 3 years old, a significant amount of children experienced eternalizing behavior problem when the mothers had smoked during pregnancy. Externalizing behaviors include overactivity, oppositional or aggressive behaviors, and these were found to occur more often in males than females with maternal smokers during pregnancy (Orlebeke et al., 1999). Additionally, data from 90,040 mother and child pairs, aged 8 months to 5 years of age, showed that maternal smoking during pregnancy leads to higher rates of internalizing behaviors such as anxiety and depression (Moylan et al., 2015) suggesting that as the child matures additional behavioral issues can occur.

In this study, the amount of time Snus-treated females spent in the closed arms of the EPM had a significantly different distribution than control females, with the distribution being shifted up. The smaller distribution in Snus treated females suggests that a higher percentage of the Snus treated females spent more time in the closed arm, which are signs of anxiogenic behaviors (Walf and Frye, 2007). Furthermore, female Snus-exposed mice had a highly significant difference in distribution of their measured anxiety index, with a higher percentage of females

experiencing increased anxiety than the female PBS-exposed mice. This differences in anxiety-like behaviors was in line with previously discussed studies, however in this study, only female mice were affected, while male mice had no significant differences between Snus-exposed and control mice. A study from 2015, which measured social behavior in mice with varying numbers of X-chromosome representing males (XY), females (XX), Klinefelter syndrome (XXY), and Turner syndrome (XO), found that the number of X-chromosomes did influence social behavior such as anxiety (Cox et al., 2015). Furthermore, females with triple X syndrome typically have increased symptoms of anxiety and depression, suggesting that anxiety and other behavioral issues may be specifically linked to the X-chromosome, leaving females more susceptible to developing anxiety from environmental factors than males (Otter et al., 2010; 2012).

Results from the Suok test also showed anxiogenic like behaviors. For female Snus-exposed mice, the latency to move from the center position was significantly higher mean, median and distribution when compared to control females. Higher latency time in mice has previously been shown to be a symptom of higher anxiety (Kalueff and Tuohimaa, 2005). Female Snus-exposed mice also had significantly higher levels of defecation during the test, a marked reaction to the fight or flight response often triggered by anxiety (Kalueff and Tuohima, 2005). The mean number of head dips was not quite significant; however, the range was shifted significantly higher in Snus exposed males. In a previous study, increased head

dips were found when mice were treated with an anxiogenic drug, suggesting that male Snus-exposed mice might have slightly higher levels of anxiety than PBS-exposed males (Benneh et al., 2018).

Another aspect of the Suok test is the ability to measure motor control functions. A study which analyzed 13,207 children aged 11, with mothers who did or did not smoke during pregnancy, showed that specifically male children had a significant loss of motor control function, particularly in their non-dominant hand than compared to children whose mothers did not smoke during pregnancy (Larsson and Montgomery, 2011). However, few studies have looked at the effect of tobacco exposure during pregnancy on motor control functions into adulthood. In the current study, exposure to Snus extract led to a significantly different mean, median, and range in the total number of missteps in females only. However, when adjusting for the total distance traveled compared to number of missteps, male mice showed to travel a shorter distance before a misstep would occur.

A novel measurement used in this study accounted for the total number of movement, head dips, side looks, and segments crossed, and compared it to the number of missteps and falls, which we call a coordination index, showed that females exposed to Snus during pregnancy had a significantly lower score than the female control mice, suggesting poorer overall coordination. However, the coordination index has not been validated yet and serves the purpose to push further investigation into coordination control due to tobacco or other harm reduction tobacco products.

In agreement with other studies that tobacco effects males and females differently, effects of the HRTP Snus were also shown here to elicit varying effects between males and females. Determining how males and females are affected differently to the same chemical may lead to more specific drugs or new legislation on environmental protections for pregnant women.

3.6 Conclusion

Over the years, conventional tobacco use has been steadily declining. However, usage of other products such as non-combustible tobacco, “light” cigarettes, and most recently e-cigarettes, collectively known as HRTPs, have been increasing. Part of the allure is that these products are marketed or generally believed to be safer than conventional cigarettes. However, the effects of using HRTPs requires further research to be able to confer them as safer products. Usage of HRTPs in pregnant women is of major concern, because while these products may be safer than conventional cigarettes, there may be unknown consequences that affect the developing fetus. Here we show that exposure to Snus during pregnancy in mice can lead to increased anxiety, as well as potentially lead to motor control issues later in life.

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4 Conclusion

4.1 Summary and concluding remarks

Health concerns regarding conventional tobacco products are well known by the public, however the use of harm-reduction tobacco products has not been studied enough to determine whether these products are “safer”. To call a product safer, it must be clear what about the product is safer. Non-combustible products may have lower carcinogens but may also negatively affect the health of a fetus. The tobacco industry, as well as government, have a duty to protect and inform the public of all health consequences from legal goods, especially tobacco products. A significant decrease in the use of conventional cigarettes has occurred over the past few decades, however other “reduced-tar” tobacco products such as “light” cigarettes, non-combustible tobacco products, and e-cigarettes have

consequentially risen in consumption. A national study in the U.S. regarding public health conceptions of e-cigarettes and conventional cigarettes suggests that 40% of the public thinks that e-cigarettes are less harmful (Kiviniemi; 2015). Therefore, it is up to researchers to inform both the government and the public of the full spectrum of health issues caused by HRTPs. In this thesis, I have discussed two very different outcomes of HRTP use; behavioral outcomes and mitochondrial health. The behavioral study found that smokeless tobacco may have effects, which are sex specific, finding that female mice are more likely to be affected by Snus than males. This came from evidence that female mice exposed to Snus have reduced motor control, a higher latency to start, increased number of missteps and a lower coordination index than males. Male mice exposed to Snus also showed negative effects such as having missteps occur in a shorter distance than untreated, but overwhelmingly these results pointed to female mice being more susceptible to the negative behavioral and motor control effects of Snus. As the mice were only exposed twice to the Snus extract *in utero*, it is significant to note that exposures during an important stage of development, was able to cause significant changes which lasted into adulthood. With mitochondrial morphology, it was shown that both the conventional, Camel, and harm reduction product, Camel Blue, led to changes in the mitochondrial network. Multiple morphological defects were found to occur only within the harm reduction product including the number of individual mitochondria, the nuclear area, and the footprint normalized to the nuclear area, while the number of networks, mean branch length, number of

branches per network and mitochondrial footprint were affected by both products. The supplementation of ascorbic acid in both the Camel and Camel Blue exposures had an overall rescue effect to the network morphology, while Caspase inhibitors differentially effected the morphology with a pattern consistent with the qPCR array, suggesting that mitochondrial health is at least partially affected through Caspase 9 in the conventional Camel and through Caspase 4 in Camel Blue-treated cells. These two caspases function in different arms of the apoptotic cascade and suggest that stress is triggered differently in these two exposures.

The major outcome of these studies is that both conventional and HRTPs negatively affect different aspects of health, with neither product standing out as a safer alternative. In 2014, a study estimated that smokeless tobacco products manufactured in the U.S. would have a 90% harm reduction over conventional cigarettes (Nutt; 2014). The criteria used to rate overall harm include things like morbidity, dependence, injury, crime, environmental damage and economic cost, however studies like this are highly mispresenting the range of damage that can occur from tobacco use. Tobacco industry leaders, along with the government, need to be specific when suggesting which harm is reduced.

While the U.S. legislation is currently fighting some concerns of HRTPs such as prohibiting the sale of flavored e-cigarettes which target minors, the fight to ensure the publics' awareness of the deleterious effects of both conventional and HRTPs must continue.

4.2 References

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