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Glyphosate is the functional component of the herbicide, RoundUp®. Although considered safe for humans by the US Environmental Protection Agency (EPA), it has been classified as "probably carcinogenic to man" by the International Agency for Research on Cancer (IARC). As such, the cellular effects of glyphosate require further study. This study was carried out to examine the role that glyphosate plays in altering human osteoblast cell function. We examined the effects of glyphosate (0.0007, 0.007, and 0.07 mg/ml) on human osteoblast cells (hFOB 1.19) for changes in proliferation rate, level of oxidative stress (ROS), glutathione (GSH) generation, and the expression of genes related to osteoblast differentiation and DNA methylation. This study was carried out to examine the role that glyphosate plays in altering human osteoblast cell function. Chronic glyphosate exposure caused a significant increase in proliferation in hFOB 1.19 in a dose dependent manner. The lower concentrations of exposure had the greatest effect on proliferative rate, with 0.007 mg/ml having the most pronounced effect. Levels of cellular ROS and GSH remained unchanged following chronic exposure. Expression of the transcription factors Osterix and RUNX2, markers for osteoblast differentiation, showed no significant change relative to the control. Similarly, no significant change was observed in Osteocalcin, a bone-specific protein synthesized by osteoblasts and early marker of *in vitro* osteogenic differentiation. However, a trending increase was observed at the lowest glyphosate concentration of 0.0007 mg/ml. While no significant changes were observed in these markers for differentiation, a significant increase in osteoblast

mineralization was observed at 0.0007 and 0.007 mg/ml. Further, by Day 7 of treatment, alkaline phosphatase activity was significantly increased across all treatment groups, indicating that glyphosate enhances osteoblast differentiation. The expression of each DNA methyltransferase (DNMT1/3a/3b), proteins that catalyze the addition of methyl groups to DNA, remained unchanged across all treatment groups. Finally, a significant increase was observed in global DNA methylation at 0.0007 mg/ml, suggesting that glyphosate is an effector of DNA methylation. While our current research is ongoing, the present findings indicate that glyphosate significantly alters osteoblast proliferation and differentiation in a dose dependent manner, however more research must be conducted to elucidate the molecular basis for these changes. This study contributes to the ongoing research into the potentially detrimental effects of glyphosate exposure on human health and provides a platform for future studies.

THE EFFECTS OF CHRONIC GLYPHOSATE EXPOSURE

ON OSTEOBLAST CELL FUNCTION

by

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APPROVAL PAGE

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Herbicide exposure has been linked to various human diseases. Glyphosate, the primary chemical component of the herbicide RoundUp®, is receiving increased scrutiny after it was found to be responsible for a case of non-Hodgkin's lymphoma in a recent lawsuit (Benbrook, 2020). Moreover, it has been detected in common breakfast cereals such as Cheerios and Quaker Oats, as well as many common beer and wine products (Cook, 2019), indicating broad and consistent exposure. Despite the global widespread use of glyphosate, the mechanistic pathways in which glyphosate alters cellular function are not well defined. Current research suggests that exposure to glyphosate leads to an increase in the generation of reactive oxygen species (ROS), perpetuating cellular oxidative stress. Increased ROS is known to cause DNA damage and negatively affect proteins and lipids. In addition, alterations to the epigenome, more specifically, increased DNA methylation, may occur in response to increased oxidative stress, leading to changes in gene expression.

The goal of this research is to further investigate the cellular consequences of chronic glyphosate exposure. A normal osteoblast cell line hFOB1.19 was chosen as a model to allow for studying how glyphosate exposure can impact not only basic cell

functions but also cellular differentiation. In addition, there are no published results of glyphosate effects on osteoblast cells and differentiation. We hypothesized that hFOB1.19 cells chronically exposed to glyphosate display altered levels of ROS and glutathione, changes in global genomic DNA methylation, and modifications in the level of osteoblast differentiation markers.

Glyphosate Use

Glyphosate is a broad-spectrum organophosphorus herbicide and is the primary chemical component Roundup®. It is widely available in many chemical forms for both agricultural use and use in home gardens. In plants, glyphosate functions to inhibit the shikimate pathway, a pathway responsible for the synthesis of the amino acids phenylalanine, tyrosine, and tryptophan. Through the inhibition of 5-enolpyruvylshikimate-3-phoaphate, the essential downstream amino acids are no longer synthesized in growing plants, eventually leading to plant death. It is a pathway that is unique to plants and not found in the mammalian genome (NPIC, 2019).

Since the development of "Roundup Ready" crops in 1996, glyphosate has become the most widely used herbicide globally. A recent study notes that from 1974 to the present, 1.6 billion kilograms of glyphosate has been applied in the United States, two-thirds of which was applied in the last 10 years (Benbrook, 2016). This accounts for nearly 20% of estimated global glyphosate use (8.6 billion kilograms). Despite the availability of "Roundup Ready" crops, the excessive use of glyphosate has led to

resistant weeds, requiring more frequent application of herbicides to crops and gardens. Through this widespread use, detectable levels of glyphosate have been identified in soils, groundwater, and precipitation and recently cereals, beers, and wines (Battaglin *et al.,* 2014). This indicates that human exposure to glyphosate is not limited to those who work in agricultural industries.

The current regulatory requirements for the applications of glyphosate are nearly 30 years old. Despite the fact that the Environmental Protection Agency (EPA) has established an "acceptable" exposure level of 0.0007 mg/ml, the World Health Organization's International Agency for Research on Cancer (IARC) has recently marked glyphosate as "probably carcinogenic to man" (IARC, 2015). As a result of increased scrutiny, the EPA reviewed the carcinogenic potential of glyphosate in 2016 and concluded that it was "not likely to be carcinogenic to humans", only one year later. These designations have reignited a need for continued research into glyphosate's effects on human health.

Glyphosate – Organismal and Cellular Effects

The organismal and cellular effects of glyphosate have been studied broadly. While glyphosate is currently considered a non-toxic herbicide due to its low LD50, multiple studies have linked glyphosate exposure to changes in cellular function. In 2013, glyphosate was shown to increase the proliferative rate of human breast cancer cells T47D through the induced activation of estrogen response element transcription activity.

This suggests that glyphosate has a potential effect on proliferative rate, serve as an endocrine disruptor, and effect gene transcription at environmentally relevant concentrations (Thongprakaisang *et al*., 2013). A similar study into the effects of glyphosate on cellular function showed that a commercial formulation of glyphosate inhibits the proliferation of fibroblasts, indicating that the observed effects may be cell type specific (Martini *et al*., 2012). And more recently, glyphosate exposure was found to cause a disturbance in the morphology of thyroid cell structure in Wistar rats, again showing its effect as an endocrine disruptor (Hamdaoui *et al*., 2020). Relevant to our current study, a perturbation of bone metabolism was observed in this study, revealing that glyphosate exposure may influence bone cells specifically. They observed the thinning and discontinuity of bone trabecular, indicating that there may be an effect on osteogenesis.

Glyphosate – Effects on Human Health and Cancer

Various human cell lines have been used as a resource for investigating the effects of glyphosate exposure. As early as 1980, the toxic effects of glyphosate were evaluated in human lymphocytes (Vyse *et al*., 1980). In this study, they focused on the Sister-Chromatid Exchange index (SCE), a means of quantifying the mutagenic effect of glyphosate. While they were able to show a significant increase in the SCE index, it was only observable at higher levels of exposure (0.1 mg/ml). Within the last decade, many more studies were conducted on the genotoxic potential of glyphosate in lymphocytes. A

2009 study observed the effects of glyphosate at environmentally relevant concentrations (3.5 μg/ml); those likely to be observed when used occupationally or residentially (Mladinic *et al*., 2009). Their results indicated that there was no effect of glyphosate on any assay applied in their study, leading them to conclude that glyphosate poses no significant risk to human health based on current usage. Contrary to these results, a more recent 2014 study was able to show glyphosate (0.7 μM – 7 μM) does induce a genotoxic effect in a dose-dependent manner on human lymphocytes (Alvarez-Moya *et al*., 2014). While the effects are not limited to a specific cell type, these studies illustrate the degree in which results may vary depending on treatment condition and chemical formulation. Because of this, there is generally no consensus on the effects of glyphosate on healthy human cells.

Within the last decade, multiple studies have evaluated the carcinogenic potential of glyphosate using varying tumor cell lines. In a study discussed previously, glyphosate caused proliferative effects on human breast cancer cells TD47 (Thongprakaisang *et al*., 2013). This was attributed to changes in estrogen receptor (ER) expression as a result of 48-hour exposure to glyphosate. A 2019 study investigated gene expression changes of common canonical pathways in both ER positive and ER negative breast cancer cell lines (Stur *et al*., 2019). They found that both cell lines displayed changes in the expression patterns of 11 canonical pathways, including those responsible for cell cycle and DNA damage repair. As these changes were seen in both ER positive and ER negative cell lines, the authors concluded that the dysregulation was the result of a multitude of molecular and cellular effects, and not solely endocrine disruption. The effects on cell

cycle and DNA damage repair ultimately were found to lead to the accumulation of mutations and cell death.

There are many recent studies investigating the link between glyphosate exposure and cancer development. In 2019, Monsanto, the company that produces Roundup®, was found liable in a lawsuit from an individual diagnosed with non-Hodgkin lymphoma. As a cause of cancer, multiple groups have studied the link between the development of non-Hodgkin lymphoma and glyphosate exposure, none of which found a statistically significant association. In 2019, data was gathered from AgriCan, the Center for Novel Agricultural Products (CNAP), and Agricultural Health Study (AHS). This data linked country-specific crop-exposure matrices to extrapolate overexposure to many pesticides and herbicides, including glyphosate. In this epidemiological study, they did find a moderately elevated positive meta-hazard ratio ($HR = 1.36$; $p < 0.05$) between diffuse large B-cell lymphoma and glyphosate exposure (Leon *et al*., 2019). In conclusion, the link between glyphosate exposure and cancer remains debatable.

ROS Involvement in Glyphosate Exposure

Reactive oxygen species (ROS) are formed during normal cellular metabolism and have important functions in cellular signaling and homeostasis. These ROS species include the superoxide anion, peroxide, hydrogen peroxide, hydroxyl radicals, and hydroxyl ions. While reactive oxygen species are a natural byproduct of normal oxygen metabolism, during periods of environmental stress ROS levels can increase and result in

damage to cellular structure, whether it be damage to DNA, RNA, proteins, or lipid peroxidation. The effects of increased reactive oxygen species have been commonly implicated in various physiological states including cancer, infertility, and ageing. Hydrogen Peroxide, for example, is a known major contributor to oxidative damage. As superoxide leaks from mitochondria through electron leak from the electron transport chain, it will undergo dismutation into hydrogen peroxide, which is later converted into water. The conversion process from hydrogen peroxide to water is not completely efficient in a cell under basal levels of oxidative stress, allowing peroxide radicals to remain (Jastroch *et al*., 2010). During periods of increased oxidative stress, excess amounts of these ROS species can cause deleterious effects. The oncogenic properties of ROS provide an interesting model for how increased oxidative stress can facilitate cancer cell survival. Moderate amounts of ROS are required for cell cancer survival. Growth factors that drive cell-cycle progression often require ROS as a second messenger for activation (Sundaresan *et al*., 1995). This allows for a potential increase in proliferation for cancer cells.

A recent in vivo study of glyphosate on *Caenorhabditis elegans* was conducted using various concentrations of a glyphosate containing herbicide, TouchDown® (Bailey *et al*., 2018). While these studies used higher concentrations of glyphosate than we plan to use in our study, it was determined that there was a significant increase in hydrogen peroxide production, likely due to the increased oxidative stress and mitochondrial inhibition. Other studies linked DNA damage to increased oxidative stress in human peripheral blood mononuclear cells when exposed to glyphosate (Woźniak *et al*., 2018).

The same study showed an increase in sequence specific DNA methylation, specifically in the p53 promoter region. Importantly to our study, it has previously been demonstrated that the commercial formulation of Glyphosate (RoundUp®) caused an increase in levels of reactive oxygen species at dilution levels far below agricultural recommendations (Chaufan *et al*., 2014). In this study, the effects of glyphosate on oxidative stress were measured in HepG2 cell line through the use of H2DCFDA, a probe used to detect multiple forms of free radicals/reactive species (OH, ONOO-, H2O2, NO, and ROO). Typically, agricultural products are supplied in a formulation of 360 g/L of acid glyphosate (RoundUp®). This study was able to show dose-dependent cytotoxicity at a concentration of 0.04 mg/ml of acid glyphosate formulation, much lower than what would be commonly found in agricultural products, although greater than the EPA acceptable exposure level.

ROS and Epigenetic Modifications

The DNA Methyltransferase (DNMT) family of proteins work to catalyze the transfer of methyl groups (CH_3) to DNA. There are two major DNMTs we are analyzing, each of which serve a unique function. DNMT1 is responsible for maintaining methylation patterns in proliferating cells. During replication, the daughter strand lacks the methylation patterns of the parent strand. DNMT1 binds to these hemi-methylated sites, allowing the addition of a methyl group to the cytosine on the daughter strand, thus conserving the established methylation pattern. DNMT3a functions to provide de novo

methylation. These proteins do not require the same hemi-methylated strands to bind, although, they are able to act on these sites, as well as unmethylated CpG islands. This allows for genome wide de novo methylation, leading to potential gene repression. DNMT3a functions primarily during embryogenesis when the methylation patterns of the embryos are re-established.

Genomic DNA methylation has multiple functions. It is involved in X-chromosomal inactivation, imprinting, transposon silencing, and gene regulation (Jin *et al*., 2011). Increased DNA methylation within CpG islands of gene promoters inhibit gene transcription. Generally, gene associated CpG islands are unmethylated, however, abnormal DNA methylation, such as that due to changes in DNMT activity, can lead to aberrant gene expression.

Cellular damage caused by reactive oxygen species (ROS) leads to varying degenerative processes within the cell. As previously mentioned, there is a delicate balance between ROS production and scavenging under normal physiological conditions. If this balance is disturbed via the introduction of xenobiotic compounds into the cellular environment, cellular stress mounts and degenerative processes begin to occur. One of the byproducts of increased oxidative damage is the modification of DNA methylation (5-mC) patterns. The most common epigenetic variance in cancer cells is abnormal DNA methylation patterns. These DNA modifications are cancer type dependent and can manifest as global demethylation or hypermethylation (Lokk *et al*., 2014). Increased ROS has been shown to activate NF-κB and to bind and activate DNMT1 expression (Hong *et al.*, 2013). It has also been proposed that increased ROS can lead to both hypo- and

hypermethylation in precancerous cells due to increased DNMT1 activity (Wu *et* al., 2015). A more recent study has shown that demethylation in glioma tissue was a direct result of the increased reaction of hydroxyl radicals with DNA (Barciszewska *et al*., 2019), further establishing a link between ROS and epigenetic modifications.

Glyphosate – Epigenetic Effects

Previous studies have shown that glyphosate leads to a decrease in the expression of DNA methyltransferase, potentially altering global methylation patterns (Smith *et al*., 2019). In this study, *Oryzias latipes* were exposed to environmentally relevant levels of glyphosate (0.0005 mg/ml) and the expression of DNMT1 was quantified through qPCR. The expression of methylcytosine dioxygenase genes (Tet1, Tet2, and Tet3) were also quantified in this study. While DNMT1 was shown to decrease, the expression of methylcytosine dioxygenase genes increased. Presumably, these changes could potentially lead to alterations in global DNA methylation patterns.

The epigenetic effects of glyphosate exposure are sparsely documented in both in vivo and in vitro studies, though many existing studies reach similar conclusions. A recent study investigated the effects of glyphosate exposure in non-neoplastic MCF10A mammary epithelial cells (Duforestel *et al*., 2019). In this study, the MCF10A cells were exposed to low-dose glyphosate (10-11 M) chronically for 21 days. They reported a reduction in 5-mC content in chronically treated MCF10A cells, concluding that glyphosate promotes global DNA hypomethylation.

Glyphosate induced hypomethylation of DNA has also been observed in human peripheral blood mononuclear cells (PBMCs) (Woźniak *et al*., 2020). This study investigated the epigenetic potential of low-level glyphosate (0.5 μM – 100 μM) over a 24-hour period. Despite a shorter treatment period, this study found a significant decrease in global DNA methylation in all treatment groups. The results from this study seem to correlate with similar studies regarding the effects epigenetic potential of glyphosate exposure, regardless of cell line, organism, and treatment duration.

Osteoblasts as a Model for Investigating the Cellular Effects of Glyphosate

hFOB 1.19 cells are SV40 large T antigen transfected human osteoblasts. These cells provide a rapidly proliferating model system and are ideal for studying human osteoblast differentiation (ATCC 2020). hFOB 1.19 cells are well-characterized and methods for inducing differentiation are well established. The osteoblast cell lineage is subject to epigenetic modification, particularly DNA methylation changes, during transformation (Kresse *et al*., 2012). Understanding the effects of glyphosate on osteoblast proliferation and differentiation is important, as it may reveal a potentially negative effect on bone tissue dynamics with consequences to human health, outside an increased cancer risk.

Project Overview and Specific Aims

Herbicide exposure has been linked to various human diseases and conditions and there is mounting evidence that an individual's accumulated environmental exposure is reflected in their epigenome. Glyphosate has the potential to negatively affect cell viability and function, including the cell's ability to proliferate and differentiate, ultimately leading to adverse health effects. Current studies show that glyphosate exposure has the potential to increase cellular oxidative stress (Chaufan *et al*., 2014). Significantly, it has been shown that increased oxidative stress can alter DNA methylation (Duforestel *et al*., 2019). Currently, there is a lack of understanding regarding how glyphosate impacts cell function. The goal of this study is to provide experimental evidence outlining the mechanistic effect of glyphosate on cellular function and to broaden the understanding of the role that epigenetics plays these changes.

Aim 1: Determine the dose-dependent effects of chronic glyphosate exposure on cell proliferation and differentiation in the human osteoblast cell line hFOB1.19.

Aim 2: Measure the levels of oxidative stress using ROS and Glutathione in human osteoblasts hFOB 1.19 chronically exposed to glyphosate.

Aim 3: Determine changes in gene expression of DNA Methyltransferase and global DNA methylation in human osteoblasts hFOB 1.19 chronically exposed to glyphosate.

CHAPTER II

MATERIALS AND METHODS

Cell Culture and Differentiation

hFOB 1.19 SV40 large-T immortalized human fetal pre-osteoblast cells were obtained from the American Type Culture Collection (ATCC® CRL-11372™). The cells were cultured in a medium consisting of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 0.3 g/L G418 (Geneticin), 10% fetal bovine serum, and 0.1 g/L penicillin-streptomycin. The cells were grown in T-75 flasks in a humidified incubator at a temperature of 34.4°C with 5% CO_2. Growth media was renewed ever 2-3 days. The cells were allowed to reach 80-85% confluence before being split. Differentiation was induced by first growing the cells to confluency and then by adding a differentiation medium containing a mixture of 0.1 g/L ascorbic acid, 5 x 10^3 mol/L β-glycerol phosphate, 10^8 mol/L menadione, and 10^{-7} $25(OH)_2D_3$ vitamin D. The cells are grown in a 39°C incubator with 5% CO_2.

Chronic Glyphosate Exposure

A 10 mg/ml glyphosate stock was prepared in water and filter sterilized through a 0.2 mM filter. hFOB 1.19 cells were plated at 2.2 x 10^6 per 100 mm dish and treated with

0.0, 0.0007, 0.007, and 0.07 mg/ml glyphosate. The cells were allowed to grow to 85-90% confluence, collected, counted, and re-plated at the same cell density. Glyphosate was added directly to the medium at each passage. Cell cultures were maintained in humidified incubator at 34.4°C with 5% CO_2 for the duration of their exposure period of four weeks. Cells were collected, resuspended in complete medium containing 5% DMSO and stored in liquid nitrogen. These cell stocks were considered "chronically exposed" hFOB 1.19 cells and were used for the various assays.

Proliferation Assay

hFOB 1.19 cells that were chronically treated with glyphosate for at least four weeks were grown in 100 mm plates for 2-3 days. The cells were collected and each treatment group re-plated at 1 x 10^3 in 96-well plates. Each assay day and treatment group included six replicas (n=6). The day after plating, the cells were considered at Day 0. Cell numbers were determined by measuring DNA content using the CyQuant NF Cell Proliferation Assay Kit (Invitrogen™ C35006). To assay for DNA content, medium was removed from the cells and 50 µl of DNA binding dye was pipetted to each well. The cells were incubated for 35 minutes at 34.4ºC to allow binding of the dye. Fluorescence was measured at a wavelength of 485/525 nm using the Synergy™ 2 Multi-Mode Microplate reader. DNA assays were repeated on days 0, 3, 4, 5, 6, and 7. Glyphosate concentrations were maintained in the medium during the entire period. Medium was changed one time during the seven-day period. Data was analyzed by standardizing the

DNA values of each individual treatment group to the Day 0 value. This controls for slight variations in starting cell number.

Alkaline Phosphatase Assay

Alkaline phosphatase (ALP) activity was measured as a determination of hFOB 1.19 differentiation, as levels of ALP increase during this process. Cells from each treatment group were plated in 96-well plates at a density of $1x10^4$ to allow for confluence after a two-day period. A separate plate was prepared for each time point (Day 0, 3, 7). N=6 for each treatment group and day. In addition, extra wells were included for DNA assay. Differentiation medium containing β-glycerol phosphate, ascorbic acid, menadione, and vitamin D were added to the cells on Day 0 to induce differentiation, as previously described. At each time point, medium was removed from the cells and washed with 1x PBS and stored at -80ºC. An ALP assay buffer containing 10.5 mg/ml diethanolamine (DEA), 0.1% Triton X-100 was prepared. To assay for ALP activity, 80 µl of the substrate solution containing 1 tablet of p-nitrophenyl phosphate (pNPP) per 2.7 ml of assay buffer was added directly to each well of the plate. The cells were incubated for 60 minutes in the dark at room temperature. 20 µl of stop solution (100 mM NaOH) was added to each well to halt the reaction. Samples were read at O.D. 405 using the Synergy™ 2 Multi-Mode Microplate reader. DNA content was determined using the CyQuant NF Cell Proliferation assay as previously described. Fluorescence was

standardized to DNA content for each treatment group. Fold-Change was relative to Day 0.

RNA Extraction and cDNA Synthesis

For analysis of differentiating cells, the chronically exposed hFOB 1.19 cells were collected and re-plated at a density of 7.5 x 10^4 cells per well of a 6-well plate. When the cells reached confluence, the medium was replaced with differentiation medium (see Cell culture and Differentiation) plus the appropriate amount of glyphosate. At Days 0, 3, and 7, cells were washed with 1x PBS and taken-up in RNA lysis buffer (Zymo Research R1060-1-100). The lysates were frozen at -80ºC. RNA was purified from the lysates using the Zymo Quick-RNA MiniPrep kit (Zymo Research R1051). RNA isolation from the nondifferentiated chronically exposed cells followed the same procedure.

cDNA was synthesized using the SuperScript™ IV First-Strand Synthesis System kit (Invitrogen™). A total of 1 µg of RNA was used in each reaction equaling a total of 20 µl cDNA per sample. Each cDNA sample was diluted with 20 µl RNase free water. The resulting cDNA was stored at -20ºC prior to qRT-PCR analysis.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

cDNA samples from each treatment group for the different studies were used in q-PCR analysis. For osteoblast differentiation, the following were analyzed: RUNX2

(Thermo Fisher Hs01047973_m1), Osterix (Thermo Fisher Hs01866874_s1), and Osteocalcin (Thermo Fisher Hs01587814_g1) with Actin (Thermo Fisher Hs00157387_m1) used as an internal control. In a separate q-PCR study, each sample was probed for DNMT1 (Hs00945875_m1) and DNMT3a (Hs01027162_m1), enzymes required for DNA methylation. TaqMan probes were used for each reaction. The standard PCR reaction consists of 5 µl of 2x TaqMan buffer (Applied Biosystems 4304437), 3.5 µl of ddH2O, and 0.5 µl of primer, and 1 µl cDNA. A master mix for each common primer was prepared. The amplification process was carried out using the Applied Biosystems™ StepOnePlus™ Real-Time PCR system in the following protocol: 95°C for 20 seconds followed by 40 cycles alternating between 95°C for 3 seconds and 60°C for 30 seconds. To quantify expression levels, relative fold-change was determined comparing actin standardized comparative threshold (C_t) values of each treatment group to the 0.0 mg control to generate a Log2 $\Delta\Delta C_t$ value.

ROS Assay

ROS activity was measured using the chemical dichloro-dihydro-fluorescein diacetate (DCFH-DA), a fluorescent dye that measures the presence of hydroxyl, peroxyl, and multiple other reactive oxygen species (Sigma-Aldrich D6883). The compound enters the cell and becomes deacetylated and subsequently oxidized by intracellular ROS species into 2', 7' -dichlorofluorescein (DCF), which fluoresces at wavelength 485/525 nm. A 5 mM stock of DCFH-DA solution was prepared using 1x PBS. hFOB 1.19 cells

were chronically treated with 0, 0.0007, 0.007, 0.07 and 0.7 mg/ml glyphosate for a period of 4 weeks. The cells were collected and re-plated in 96-well culture plates (n=12 for each treatment group) and allowed to adhere overnight. Additional wells of the same cells for each treatment group were plated at the same time for DNA content analysis (n=12). The following day, the medium was removed, cells were washed with 1x PBS, and 100 µl of 50 mM DCFH-DA solution was added to each well. The cells were incubated at 34ºC for 30 minutes and read at 485/535 nm using the Synergy™ 2 Multi-Mode Microplate reader. To ensure equal cell number, DNA levels were quantified in the additional wells using CyQuant NF Cell Proliferation Assay kit (Invitrogen™). hFOB 1.19 cells were plated at the same density, received the same treatment, and were cultured in the same conditions as the cells assayed for ROS activity. The ROS fluorescence values were standardized to DNA content.

Glutathione Assay

Glutathione (GSH) levels were quantified through the use of the Invitrogen™ fluorometric dye monochlorobimane (Thermo Fisher M1381MP). When bound to reduced or oxidized glutathione, MCBI will fluoresce at 360/460 nm. A stock 11.05 mM MCBI solution was prepared in DMSO. Prior to adding the solution to the cells, a 50 µM dilution is prepared in 1x PBS. hFOB 1.19 cells were plated, treated, and grown under the same conditions as the cells used for the ROS assay described above (n=12). Following overnight adhesion, the cells were washed with 1x PBS and 100 µl of MCBI was added.

The cells were incubated at 34ºC for 30 minutes and read 360/460 nm using the Synergy™ 2 Multi-Mode Microplate reader. The same wells assayed for DNA content as described for the ROS assay were used for the Glutathione assay.

5-mC ELISA Assay

hFOB 1.10 cells were chronically treated with the different concentrations of glyphosate for four weeks. At 85-90% confluence, the cells were washed with 1x PBS and collected in 15 mL falcon tubes. The cells were pelleted by centrifugation, the supernatant was removed, and the cell pellets were used for isolation of genomic DNA. Pellets were stored at -80ºC until use. Genomic DNA was extracted from the cells using the Quick-DNA Magbead Plus kit (Zymo Research D4081). Levels of gDNA were quantified using a Thermo Scientific™ Nanodrop 2000. DNA methylation was quantified using the Zymo Research™ 5-mC DNA ELISA kit (D5326) and protocol. Exactly 100 ng of gDNA was used per well in the ELISA.

Alizarin Red Staining

The cells were grown to confluence at which time the medium was replaced with differentiation medium. Glyphosate concentrations were maintained during the course of the study. Cells were grown under differentiation conditions for 21 days prior to assay. The cells were plated on a 12-well culture dish at a density of 1 x 10^5 and allowed to

grow to confluence. Once confluent, growth medium was replaced with differentiation cocktail containing glyphosate. The cells were incubated for 21 days in a humidified incubator at 39ºC with 5% CO_2. The differentiation cocktail was refreshed every 3-4 days for the duration of the experiment.

After the 21-day period, the bone matrix was stained with the Alizarin Red S Staining Kit (ScienCell Research Laboratories Cat. #0223). The cells were fixed with 4% formaldehyde, stained for 30 minutes with 40 mM ARS solution, and washed 5x with diH_2O. The plates with the stained cells were stored at -20ºC until use. The cells were collected using a cell scraper in 400 µl of 10% acetic acid. All samples were heated to 85ºC for 10 minutes. A volume of 150 µl of 10% ammonium hydroxide was used to neutralize the acid. An aliquot of 150 µl of each sample was plated in triplicate on a 96-well plate and absorbance was measured at 405 nm using the Synergy™ 2 Multi-Mode Microplate reader.

Statistics

Individual assays included at least three technical replicas and for some assays 6-12 replicas. Standard error was determined for all data sets. For some assays, three independent trials were carried out and the data combined by averaging the three values. Significance was evaluated using the Student T-test with $p<0.05$ considered significant.

CHAPTER III

RESULTS

Effects of Chronic Glyphosate Exposure on hFOB 1.19 Proliferation

To evaluate the effects of chronic glyphosate exposure on non-differentiated hFOB 1.19 cell proliferation, cells that were exposed to 0.0, 0.0007, 0.007, and 0.07 mg/ml glyphosate for at least four weeks were grown in the presence of glyphosate over a 7-day period. On days 0, 3, 4, 5, 6, and 7, a set of cells (n=6) from each treatment group were stained with a fluorometric dye that binds nucleic acid. Emitted light values are proportional to the number of cells, allowing for quantification of cell proliferation over time. The values at each day were standardized to Day 0 for each treatment group. This controlled for slight variations in cell number at plating.

At Day 3, proliferation at 0.0007 mg/ml was significantly lower than our control. By Day 4, we found a similar lower rate in both 0.07 and 0.0007 mg/ml and remained significantly lower through Day 5. By Day 6, a significant increase in proliferation was observed at 0.0007 mg/ml, while 0.07 mg/ml now showed no difference from the control group. On the 7th day, there was no significant difference between control group and the 0.0007 and 0.07 mg/ml treatment groups. Interestingly, beginning on Day 4, the 0.007 mg/ml group showed a significant increase in cell proliferation that remained increased throughout the duration of the study. The increase in proliferation of the 0.007 mg/ml treatment groups was confirmed in three independent trials. These results suggest that

chronic exposure of hFOB 1.19 osteoblasts to low levels of glyphosate, including the EPA exposure limit, positively effects proliferation. Though there was an initial decrease in proliferation at 0.0007 and 0.07 mg/ml, these differences was stabilized by the end of the study, with 0.0007 mg/ml increasing significantly as it progressed. The trend in proliferation seems to be in an inverted U shape, indicating that the trend is non-monotonic.

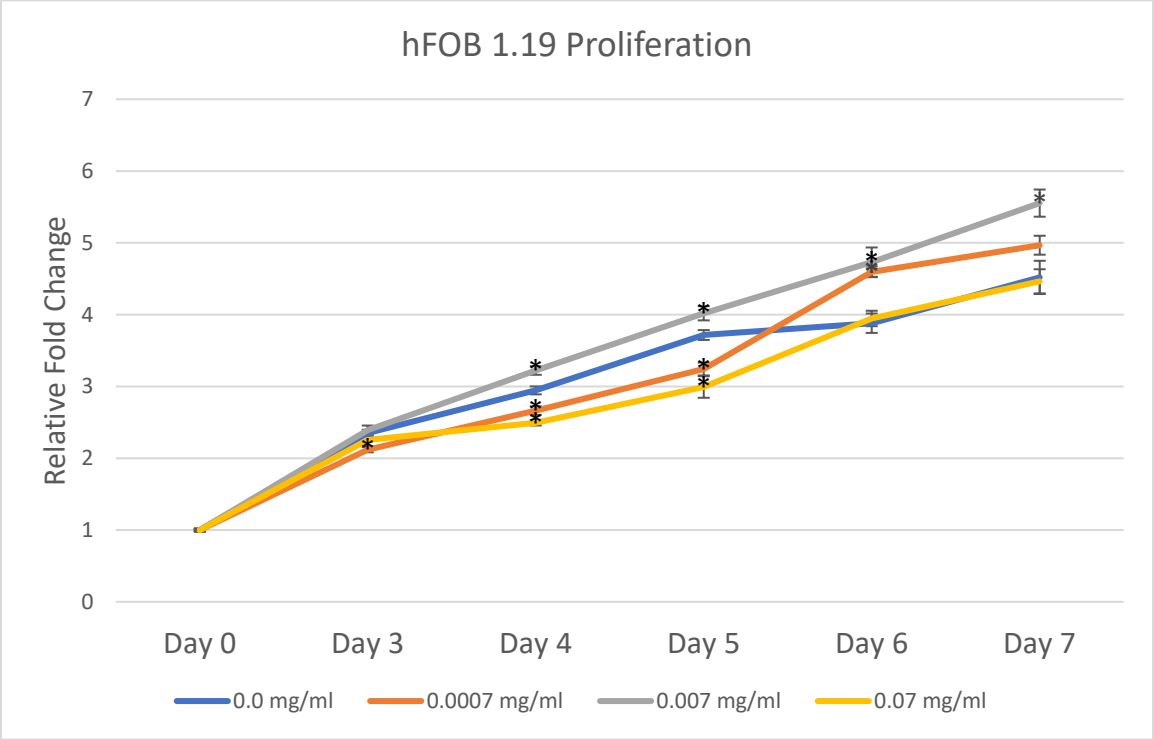

Figure 1. hFOB 1.19 Cell Proliferation Following Chronic Glyphosate Exposure. Low level, chronic glyphosate exposure has a positive effect on hFOB1.19 osteoblast proliferation. hFOB1.19 were treated with 0.0, 0.0007, 0.007, and 0.07 mg/ml for at least one month. Cells were plated at a low concentration in 96-well plates. DNA content was measured from Day 0 to Day 7, as a measure of cell number. Values are expressed relative to Day 0 for each treatment group. Asterisks indicate significance ($p<0.05$) comparing the treatment group to the control for that day. N=6 for each treatment group at each day.

Effects of Chronic Glyphosate Exposure on Osteoblast Cell Differentiation

Next we determined if chronic glyphosate exposure effected osteoblast differentiation. Three markers of differentiation were measured: level of alkaline phosphatase (ALP) activity, expression of genes associated with differentiation, and accumulation of extracellular bone matrix.

ALP Activity

We measured ALP activity in chronically exposed hFOB 1.19 osteoblasts induced to differentiate. Cells were assayed at Days 3 and 7 (Fig. 2A/2B). At Day 3, APL levels in the 0.0007 and 0.007 mg/ml treated cells are no different from the control, whereas there was a significant decrease in the 0.07 mg/ml cells. In contrast at Day 7, there is a significant increase in ALP activity in hFOB 1.19 cells exposed to glyphosate, at all experimentally relevant concentrations (0.0007, 0.007, and 0.07 mg/ml), relative to an untreated control. There was a visible dose-dependent effect on alkaline phosphatase activity on day 7, whereas the lowest concentration of glyphosate caused the greatest increase in ALP activity. These results suggest that chronic glyphosate exposure enhances osteoblast differentiation.

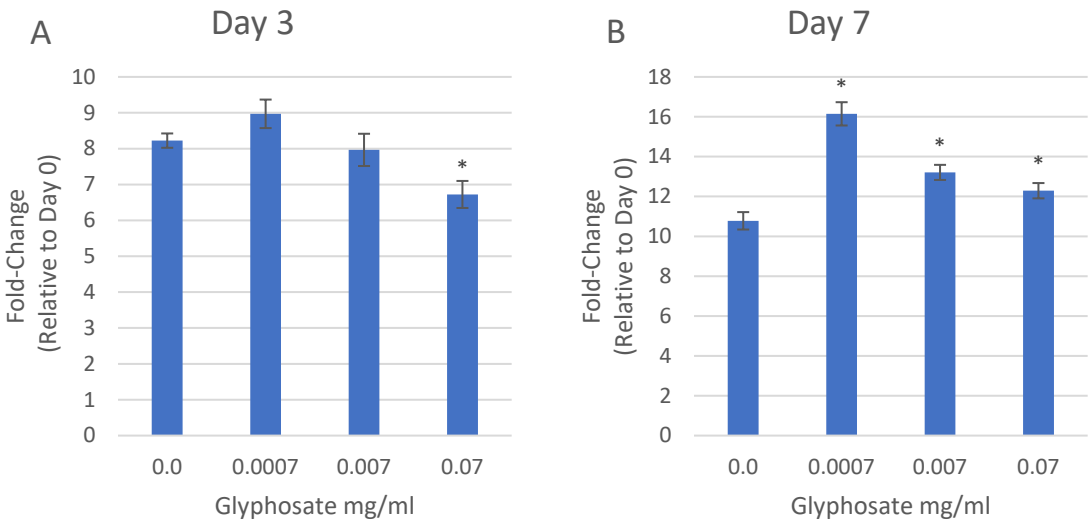

Figure 2. Low Level, Chronic Glyphosate Exposure Increases Alkaline Phosphatase (ALP) Activity in Differentiating hFOB1.19 Osteoblasts. hFOB1.19 cells exposed for at least one month to 0.0, 0.0007, 0.007, and 0.07 mg/ml glyphosate were plated under differentiation conditions. At Days 3 (A) and 7 (B) and cells were assayed for ALP activity. ALP activity was standardized to DNA content, as a measure of cell number and fold-change relative to Day 0 for each treatment group plotted. Asterisks indicate significance ($p<0.05$) relative to the control. N=12 for each treatment group.

Expression of Differentiation Associated Genes

To further determine the effects of chronic glyphosate exposure on hFOB 1.19 cells, q-PCR analysis of the expression of three genes associated with differentiating osteoblasts was performed. By Day 3, mRNA levels for RUNX2 and Osterix, transcription factors required for determination of the osteoblast lineage and early markers for osteoblast differentiation, were statistically the same between each of the treatment groups (0.0007, 0.007, and 0.07 mg/ml) relative to the control (Fig. 3A/B). However, a trend in decrease in osterix transcript levels were noted for all three glyphosate concentrations. The relative levels of Osteocalcin mRNA, a protein secreted

solely by osteoblasts, was also found not to be significantly different, comparing each treatment to the control on Day 3 or Day 7 (Fig. 3C). However, at both Days 3 and 7, there was a trend in increase in osteocalcin at the lowest glyphosate concentration of 0.0007 mg/ml.

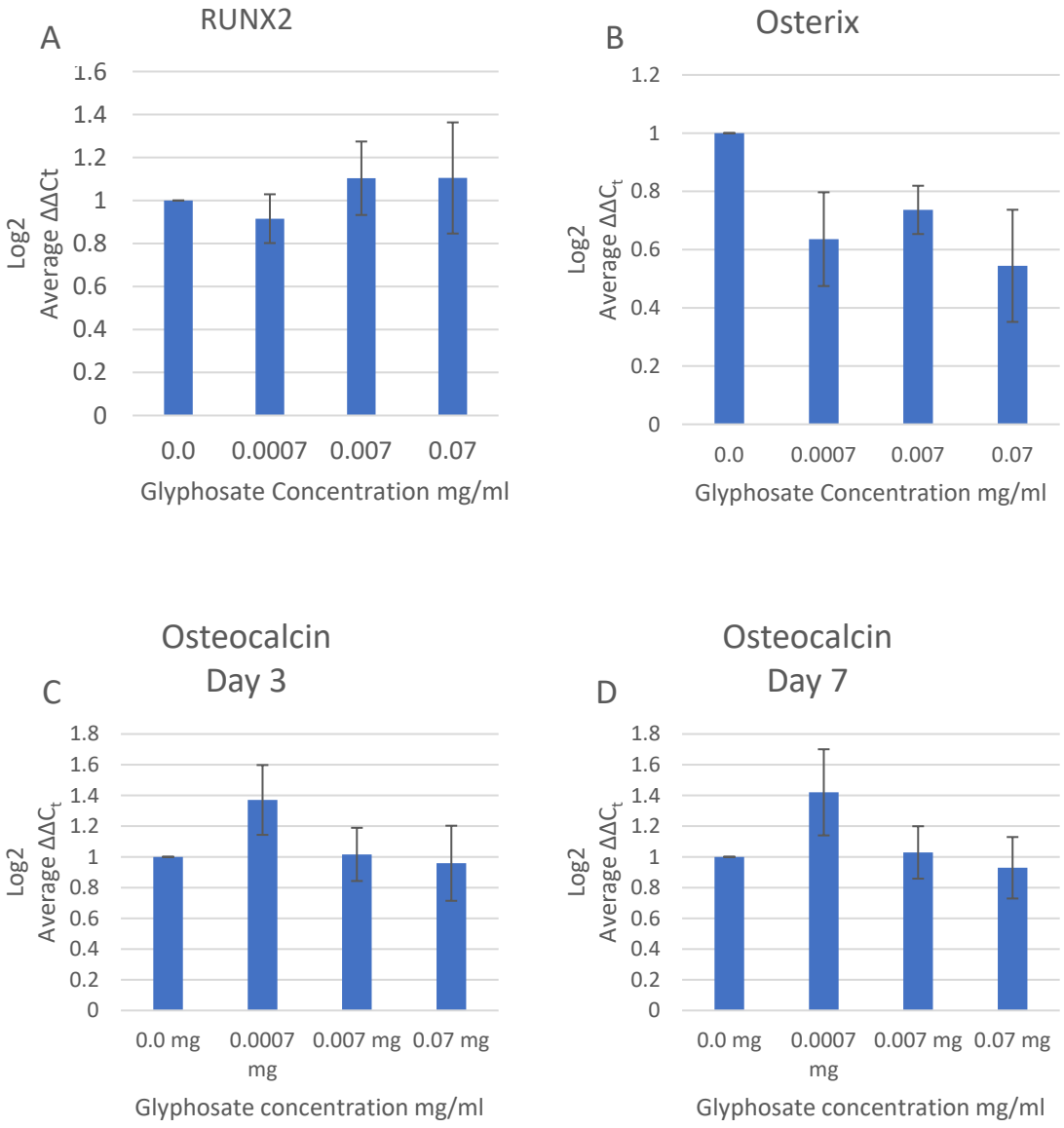

Figure 3. Low Level, Chronic Glyphosate Exposure Had No Significant Effect on the Relative Expression of RUNX2 (A), Osterix (B), or Osteocalcin(C-D). hFOB 1.19 were chronically treated with glyphosate for at least four weeks prior to the induction of differentiation. Glyphosate levels were maintained throughout the differentiation process. Relative fold-change was determined comparing actin standardized comparative threshold (C_t) values of each treatment group to the 0.0 mg control to generate a $\Delta\Delta C_t$ value. Each resulting value was divided into the control for the given day to generate fold-change relative to the control. Data are representative of three independent q-PCR assays. The average $\Delta\Delta C_t$ of the three trials were determined.

Extracellular Matrix Assay

The level of extracellular matrix calcium was assayed as an indication of advanced osteoblast differentiation. hFOB 1.19 cells were allowed to grow in the presence of glyphosate for at least four weeks. These chronically treated cells were collected, counted, and re-plated on 12-well plates (N=3) and allowed to reach confluence. Once confluent, differentiation was induced as previously described and the cells were grown for a period of 21 days. Differentiation medium was replaced every four days and glyphosate levels were maintained for the duration of the experiment. At day 21, the cells were stained with Alizarin Red S.

Extracellular calcium deposits were observed 3-weeks post osteogenic induction. Differentiated hFOB 1.19 cells stained red represent positive Alizarin Red S Staining, indicating mineralization. At Day 21, hFOB 1.19 osteoblasts showed a significant increase in mineralization at 0.0007 and 0.007 mg/ml, while there was no difference at 0.07 mg/ml relative to the control (Fig. 4). These results suggest that chronic, low level exposure to glyphosate enhances osteoblast differentiation.

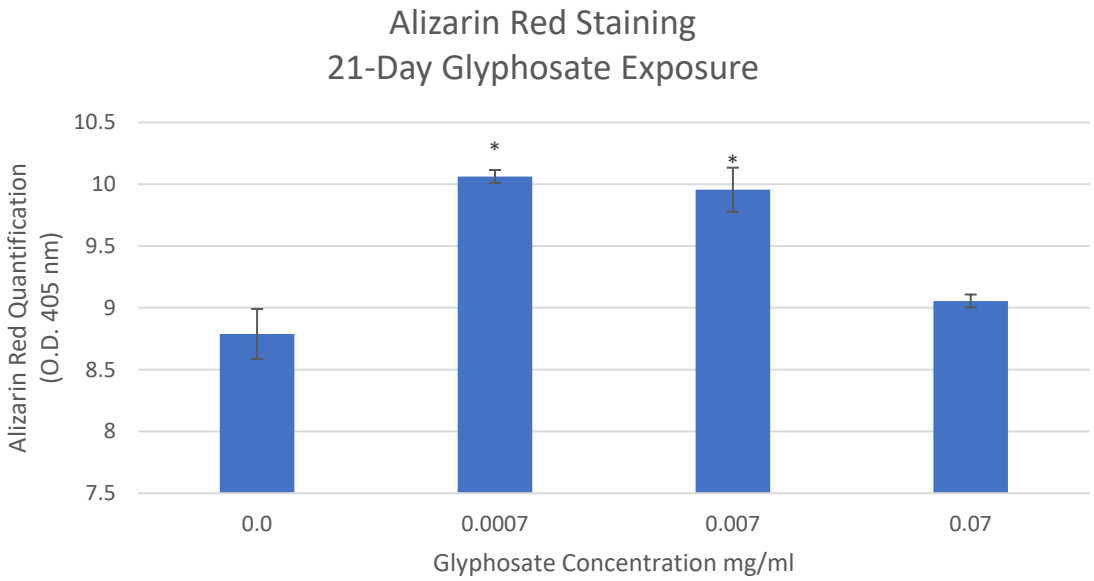

Figure 4. Differentiated hFOB 1.19 Osteoblast Mineralization Following 21-Day Glyphosate Exposure. Osteogenic differentiation was induced in hFOB 1.19 cells chronically exposed to 0.0, 0.0007, 0.007, and 0.07 mg/ml glyphosate for 4 weeks. Differentiating cells were grown for 21 days in the presence of glyphosate. The cells were assayed by Alizarin Red Staining for to measure mineralization. Shown is the average O.D. 405 nm (n=3). Asterisks indicate significance ($P<0.05$).

ROS Activity in hFOB 1.19 Cells Chronically Exposed to Glyphosate

Levels of reactive oxygen species (ROS) were measured following 4-week chronic glyphosate exposure using dichloro-dihydro-fluorescein diacetate (DCFH-DA). Because of the transitory and short-lived nature of ROS species, levels of intracellular ROS may vary between assays, however, the results from three independent assays were combined. In the current study, we found that hFOB 1.19 cells show no significant differences in ROS activity following 4-week chronic glyphosate exposure when compared to a control group (Fig. 5). DNA content of these cells was measured, and fluorescence values were standardized by DNA content to ensure equal cell number per

treatment group.

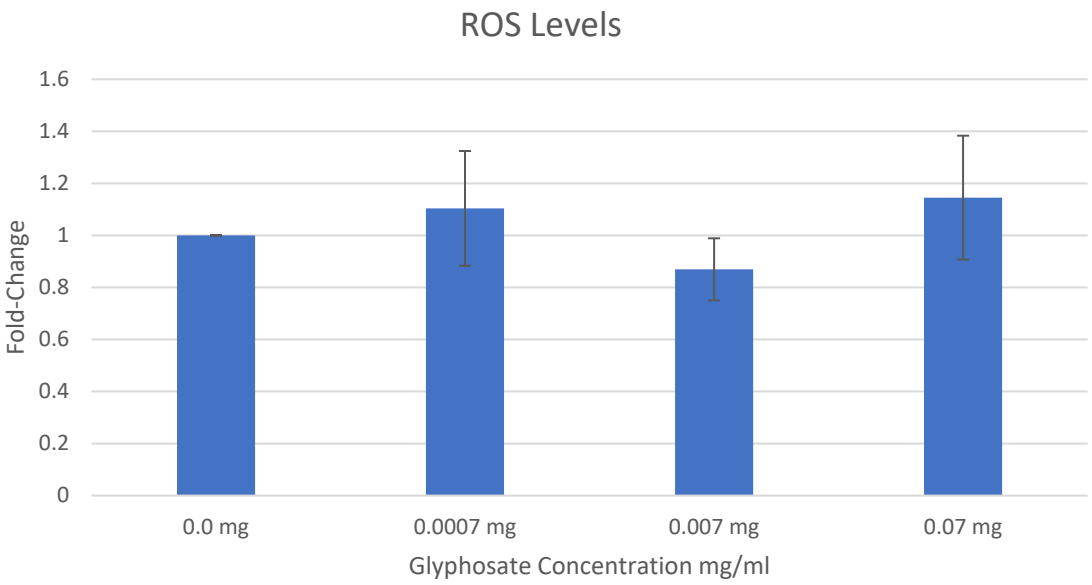

Figure 5. Levels of Intracellular ROS in hFOB 1.19 Cells Following Chronic Glyphosate Exposure. Activity of reactive oxygen species (ROS) in proliferating hFOB 1.19 cells shows no significant difference following chronic glyphosate exposure. Following 4-week chronic exposure, hFOB .19 osteoblasts were assayed for ROS activity. At concentrations of 0.0007, 0.007, and 0.07 mg/ml, glyphosate caused no significant change in ROS level. ROS activity was standardized to DNA content as a measure of cell number. This data is the result of three independent assays (n=12 per assay).

Glutathione Activity in hFOB 1.19 Cells Chronically Exposed to Glyphosate

To further quantify the effect of chronic glyphosate exposure on hFOB 1.19 cells, glutathione (GSH) activity was assessed. It is possible that increased glutathione scavenges excess ROS due to glyphosate exposure. Similar to ROS, glutathione has an ephemeral nature, indicating that levels may vary at different time points. In the current study, we observed no significant change in GSH activity in hFOB 1.19 cells following

4-week chronic glyphosate exposure in all treatment groups (0.0007, 0.007, 0.07 mg/ml) when compared to the control group. DNA content of these cells was measured, and fluorescence values were standardized by DNA content to ensure equal cell number per treatment group.

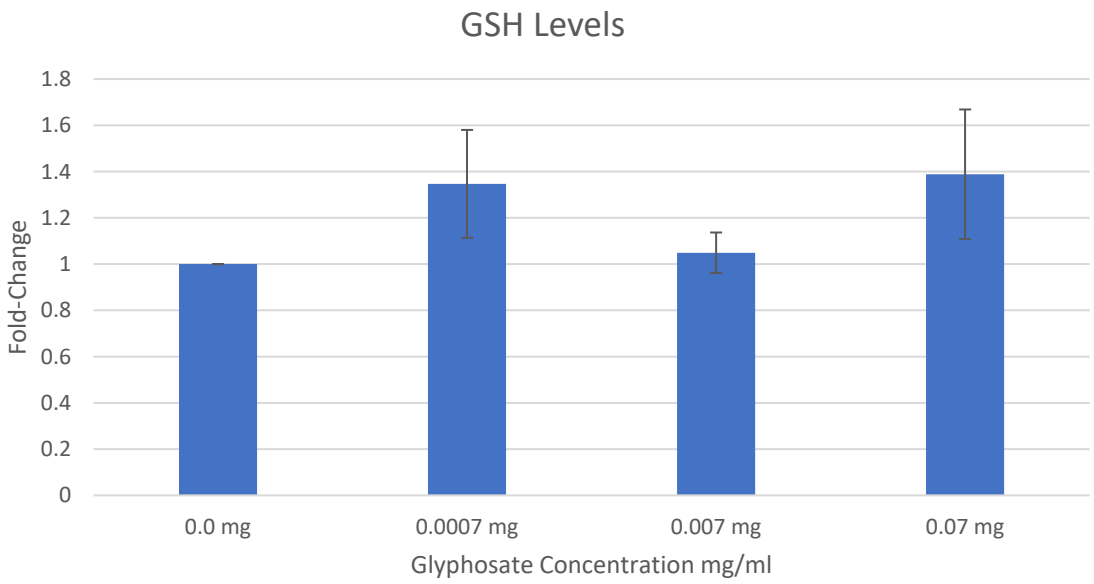

Figure 6. Levels of Intracellular Glutathione in hFOB 1.19 Cells Following Chronic Glyphosate Exposure. hFOB 1.19 cells were treated with 0.0, 0.0007, 0.007, and 0.07 mg/ml glyphosate for a period of 4-weeks. Cells were plated at alow concentration in 96-well plates. DNA content was quantified as a measure of cell number and fluorescent values were standardized by DNA content. The activity of glutathione (GSH) in hFOB 1.19 human osteoblast cells shows no significant change following 4-week chronic glyphosate exposure relative to the control.

The Effects of Chronic Glyphosate Exposure on DNMT Expression and Global DNA Methylation

To analyze the potential epigenetic effects of chronic glyphosate exposure on hFOB 1.19 cells, qPCR analysis of the expression of genes associated with DNA methylation. DNMT1 and DNMT3a are part of a family of enzymes that facilitate the transfer of a methyl group to DNA, potentially altering gene expression; DNMT1 is responsible for maintaining methylation patterns, and DNMT3a is required for "de novo" methylation. Following 4-week exposure to glyphosate, no significant changes were found in the expression of DNMT1 and DNMT3a in all treatment groups (Fig. 7).

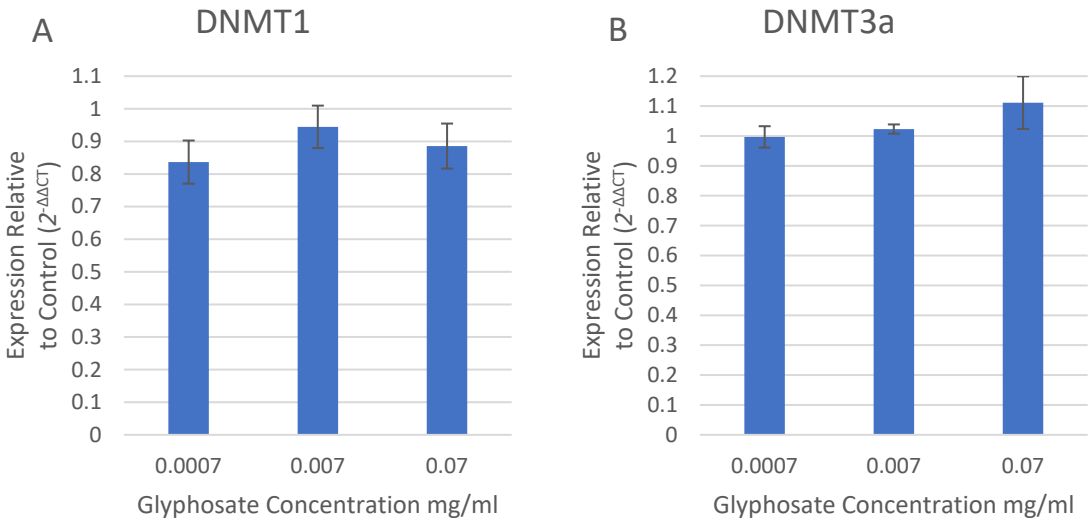

Figure 7. Gene Expression of Epigenetic Markers in hFOB 1.19 Osteoblast Cells Following Chronic Glyphosate Exposure. hFOB 1.19 cells were treated with 0.0, 0.0007, 0.007, and 0.07 mg/ml glyphosate for a period of at least 4 weeks. RNA was isolated, converted to cDNA, and probed for DNMT1 (A) and DNMT3a (B) transcripts using TaqMan® primers. Actin was used as a housekeeping gene. Values are reported relative to the control using the $2^{-\Delta\Delta CT}$ method. Low Level, chronic glyphosate exposure had no significant effect on the expression of DNMT1 or DNMT3a.

Despite finding no significant change in DNMT expression, we sought to further investigate the epigenetic effect of chronic glyphosate exposure on global DNA methylation. The gDNA used in this experiment was extracted from hFOB 1.19 cells exposed for a period of at least four weeks at the experimental concentrations previously defined. A significant increase in global DNA methylation was only observed at our lowest concentration of exposure, 0.0007 mg/ml. Among the remaining treatment groups, no significant changes in global DNA methylation were observed relative to the control (Fig. 7).

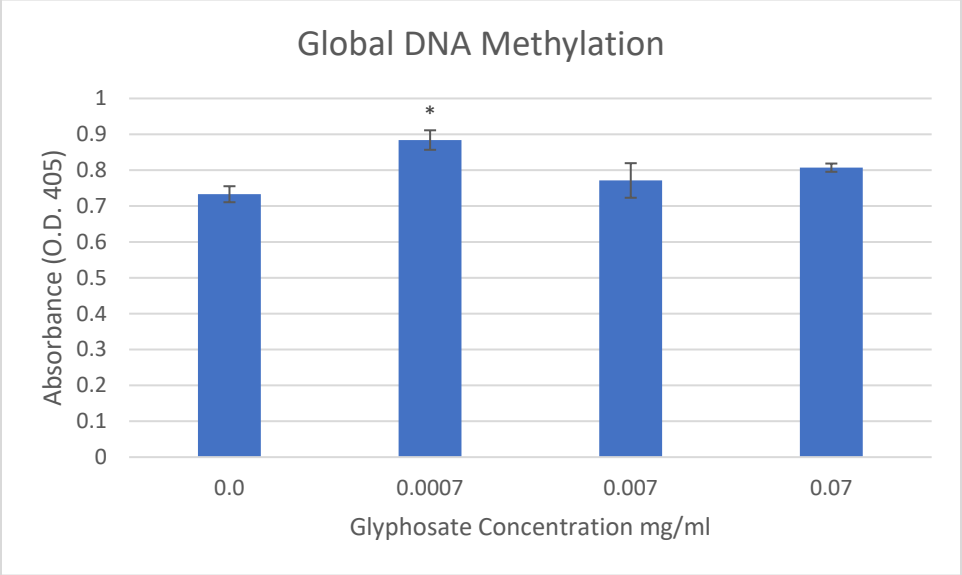

Figure 8. Global DNA Methylation in hFOB 1.19 Cells Chronically Exposed to Glyphosate. hFOB 1.19 cells were treated with 0.0, 0.0007, 0.007, and 0.07 mg/ml glyphosate for a period of 4-weeks prior to gDNA extraction. gDNA was quantified to ensure equal amounts were used for each sample. Each sample was assayed in triplicate. Following 4-week chronic exposure, a significant increase was observed at 0.0007 mg/ml relative to the control. Asterisks indicate significance ($P<0.05$).

CHAPTER IV

DISCUSSION

Herbicide exposure has been implicated in a wide range of human health problems ranging from developmental effects to cancers. More recently, there has been a call for greater research on the role of glyphosate exposure, the most widely used commercial and agricultural herbicide, on human health. In non-human organismal models, glyphosate has been shown to disrupt maturation in rats and fish (de Liz Oliveira Cavalli *et al*., 2013; Zebral *et al*., 2017) and to increase developmental abnormalities, including craniofacial structures and brain development in *Xenopus laevis*)Paganelli *et al*., 2010; Cattani *et al*., 2017). The potential carcinogenic effects of glyphosate exposure continue to be investigated and it has recently been deemed "probably carcinogenic to man" by the World Health Organization's International Agency for Research on Cancer. Because of the widespread use of glyphosate, detectable levels have been identified in groundwater and precipitation as well as household consumables such as beers, wines, and cereals (Battaglin *et al*., 2014). Despite the potential hazards associated with glyphosate, it remains the most widely used herbicide globally, underlining the importance in determining the molecular effects of glyphosate exposure on human health.

The molecular effects of glyphosate exposure are not well defined and have been noted to be cell type specific. A commercial formulation of glyphosate was found to inhibit the proliferation of human fibroblasts at environmentally relevant concentrations,

while increasing the proliferative rate in human breast cancer cell lines (Martini *et al*., 2012; Thongprakaisang *et al*., 2013). It is important that we understand the effects of chronic glyphosate exposure on cell proliferation as it has implications in human health. Currently, there is little research into the molecular effects of chronic glyphosate exposure in human osteoblast cells. In the present study, we investigated the role of low-level chronic glyphosate exposure over a period of four weeks.

Low Level Chronic Glyphosate Exposure Enhances Osteoblast Proliferation

We demonstrated that during a 7-day proliferation study, concentrations of 0.0007 mg/ml and 0.007 mg/ml positively affected proliferation in hFOB 1.19 cells (Fig. 1). This finding is consistent with previous studies that identified glyphosate as an effector of cell proliferation (Martini *et al*., 2012; Thongprakaisang *et al*., 2013). While our results suggests that chronic glyphosate exposure is a positive effector of proliferation in human osteoblasts, previous studies show diverging effects on proliferation in an acute treatment setting. In 2012, Martini *et al.* showed that low level glyphosate exposure to glyphosate can inhibit proliferation fibroblast cells. In contrast, Throngprakaisang *et al.* demonstrated that low level glyphosate exposure caused an increase in cell proliferation in breast cancer cells, indicating that the effect may be cell line dependent. One potential consequence of enhanced osteoblast proliferation is an increase in osteoblast population within the bone tissue, leading to potentially detrimental health effects.

Low Level Chronic Glyphosate Exposure Enhances Osteoblast Differentiation

We also investigated the effects of chronic glyphosate exposure on the expression of three genes associated with human osteoblast differentiation: Osterix, RUNX2, and Osteocalcin. RUNX2 and osterix are transcription factors required for determination of the osteoblast lineage and are typically early markers for osteoblast differentiation. We report no significant difference in the expression of these genes relative to the control (Fig 3A-B). Osteocalcin is a protein that is uniquely secreted by osteoblasts and is typically found to increase as these cells differentiate. While we saw no significant difference between our treatment groups and our control, we saw a trending increase in osteocalcin expression at our lowest concentration of exposure, 0.0007 mg/ml (Fig. 3C-D). Further replications of this study will be necessary to determine if this trend is significant. If so, the results would suggest that chronic glyphosate exposure enhances osteoblast differentiation.

We further categorized glyphosates effect on differentiation by measuring alkaline phosphatase (ALP) activity. ALP is considered to be one of the most commonly accessible identifiers of osteoblast differentiation (Hashemibeni *et al*., 2013). ALP is expressed early in the differentiation process and is necessary for the mineralization of bone (Jafary *et al*., 2017). It reasons that greater ALP activity has greater potential to impact bone formation. Here, we report a dose-dependent effect on hFOB 1.19 ALP activity at Day 7 of osteoblastic differentiation, whereas the lowest concentration of glyphosate seems to cause the greatest increase in ALP activity (Fig. 2). Despite seeing no significant changes in the expression of our differentiation associated genes, a

significant increase in ALP activity was detected at Day 7 of osteogenesis, suggesting that chronic glyphosate exposure enhances osteoblast differentiation. It is possible that if RUNX2 and Osterix are not effected by glyphosate, that it is possible that the effect on differentiation is a result of glyphosate effecting the ALP and Osteocalcin genes themselves. Furthermore, it is plausible that the effect could be epigenetic. We observed increased methylation at 0.0007 mg/ml in our 5-mC ELISA assay. Previous studies have indicated that the Osteocalcin promotor is susceptible to epigenetic modification, thus effecting osteoblast differentiation (Villagra *et al*., 2002).

We also analyzed osteoblast differentiation at 21 days post-induction. At this time, osteoblasts should be fully differentiated into osteocytes and secreting bone matrix. Using the Alizarin Red assay, the amount of mineralization was compared. In this study, we allowed our chronically treated osteoblasts to differentiate for a period of 21 days. Similar to ALP activity, we noted a significant increase in mineralization at 0.0007 and 0.007 mg/ml glyphosate (Fig. 4). These data indicate that chronic glyphosate causes an increase in osteoblastic maturation as early as 7 days into the differentiation process, which was reflected at late stage differentiation.

No Effect of Chronic Glyphosate Exposure on Osteoblast ROS or Glutathione Levels

Literature suggests that glyphosate exposure can alter cellular ROS production, some of which are at levels well below agricultural recommendations (Bailey *et al*.,

2018; Chaufan *et al*., 2014]. Chaufan *et al.* reported that glyphosate exposure caused a dose-dependent increase in oxidative stress at concentrations as low as 0.04 mg/ml, much lower than what would commonly be found in agricultural products. The concentrations in our study range from 0.0007 to 0.07 mg/ml glyphosate. We reported no change in ROS activity in hFOB 1.19 cells following chronic glyphosate exposure. Glyphosate is available in many formulations. While the studies above used commercial formulations of glyphosate acid, our study investigated concentrated glyphosate in ddH_2O, possibly indicating the differences in results.

Antioxidants serve a critical role in cellular redox balancing. As oxidative stress mounts in a cell, the expression of antioxidants, such as glutathione, increases accordingly to return intracellular ROS to a basal level. Previously mentioned studies indicated that glyphosate exposure alters levels of cellular ROS, which could potentially alter cellular glutathione levels. Evaluation of hFOB 1.19 cells chronically exposed for 4 weeks to low levels of glyphosate showed no significant change in glutathione levels (Fig. 6). Xenobiotic compounds are known to induce oxidative stress (Henkler *et al*., 2010). A potential explanation for these data could be indicated by the ephemeral nature of ROS and glutathione. There is the possibility that these cells have adapted to the increased environmental stress and are well suited to deal with the increased ROS generation. Further time-course studies are needed to investigate this hypothesis.

Low Level, Chronic Glyphosate Exposure Enhances Global DNA Methylation; No Effect on DNMT Expression

As previously indicated, a byproduct of increased oxidative stress is the modification of DNA methylation (5-mC) patterns. The most common epigenetic variance in cancer cells is abnormal DNA methylation patterns. Recent studies have shown that demethylation in glioma tissue was a direct result of increased reaction of hydroxyl radicals with DNA (Barciszewska *et al*., 2019), however this effect has not been investigated in osteoblastic cells. Smith *et al.* (2019) have shown that glyphosate leads to a decrease in the expression of DNA methyltransferase in a fish model. Generally, DNA methyltransferase proteins work to catalyze the transfer of methyl groups to DNA, though the epigenetic effects of glyphosate are sparsely documented. In the present study, we show that the expression of DNMT1 and DNMT3a show no significant change following chronic glyphosate exposure in human osteoblast cells (Fig. 7). Though DNMT expression remains unchanged, glyphosate still has the potential to alter the epigenome. The methylcytosine dioxygenase (TET) genes function to remove methyl groups from DNA and have been noted to increase following glyphosate exposure in previous studies (Smith *et al*., 2019). Further gene expression analysis is required on osteoblasts to determine if the effect is seen in our cell line.

Experimental studies have shown altered epigenetic marks such as changes in global DNA methylation in response to glyphosate exposure. Hypomethylation has been documented in human peripheral blood mononuclear cells as a byproduct of acute glyphosate exposure (Woźniak *et al.*, 2020). Despite a shorter treatment period, this study

found a significant decrease in global DNA methylation in response to low-level glyphosate exposure. Our present study investigated the effects of chronic low-level exposure on global 5-mC. Human osteoblasts were differentiated in the presence of glyphosate for 21-days prior to assay. We found a significant increase in global DNA methylation at 0.0007 mg/ml relative to the control (Fig. 8). No significant changes were observed in the remaining treatment groups. These results are in contrast with many studies that previously discussed. A possible explanation would be the use of differing cell types. Another important consideration is that measurements of global methylation do not reveal sequence specific methylation changes. Increased methylation within gene promoter CpG islands could affect gene expression, whereas in other genomic locations, such changes may have no effect. DNA methylation and epigenetic patterning is a complex process and is known to vary within cell populations of the same type (Rakyan *et al*., 2008). Though these data indicate that chronic glyphosate exposure has no effect on the expression of DNMTs, which are responsible for DNA methylation patterning in human osteoblasts, additional studies on TET gene expression would be beneficial for confirming the epigenetic effects of chronic glyphosate exposure. In addition, analysis of sequence-specific DNA methylation and gene expression using RNAseq analysis in chronically glyphosate exposed cells would provide a more complete understanding of the possible epigenetic effects of glyphosate exposure. Interpretation of these data need to take into consideration the complex nature of cellular epigenetics.

The effects of glyphosate exposure are varied and depend on cell type, length of exposure, and concentration. The chronic effects of glyphosate exposure are not well

documented. In the present study, we have shown that chronic low-dose glyphosate exposure has a positive effect on hFOB 1.19 proliferation. The lowest two levels of exposure in our study (0.0007 and 0.007 mg/ml) had the greatest enhancing effect on proliferation, ALP activity, and bone mineralization. The lowest level of exposure (0.0007 mg/ml) showed the greatest significant increase on global DNA methylation. Similarly, we also saw a trending enhancement of Osteocalcin expression, though not at a significant level. It was common throughout our study that the greatest effects of glyphosate exposure occurred at the lowest concentrations of exposure, including the EPA acceptable limit of 0.0007 mg/ml. Hence, environmentally relevant concentrations have the potential to alter bone dynamics by increasing osteoblasts and enhancing differentiation. This study provides insight into the effects of chronic glyphosate exposure on a human cell line model and provides a platform for future studies.

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