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# Mechanistic insight into the effects of Aryl Hydrocarbon Receptor activation on osteogenic differentiation

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ABSTRACT

While inhibition of bone healing and increased rates of pseudarthrosis are known adverse outcomes associated with cigarette smoking, the underlying mechanisms by which this occurs are not well understood. Recent work has implicated the Aryl Hydrocarbon Receptor (Ahr) as one mediator of the anti-osteogenic effects of cigarette smoke (CS), which contains numerous toxic ligands for the Ahr. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) is a high-affinity Ahr ligand frequently used to evaluate Ahr pathway activation. The purpose of this study was to elucidate the downstream mechanisms of dioxin action on bone regeneration and investigate Ahr antagonism as a potential therapeutic approach to mitigate the effects of dioxin on bone. Markers of osteogenic activity and differentiation were assessed in primary rat bone marrow stromal cells (BMSC) after exposure to dioxin, Ahr antagonists, or antagonist + dioxin. Four Ahr antagonists were evaluated: α-Naphthoflavone (ANF), resveratrol (Res), 3,3′-Diindolylmethane (DIM), and luteolin (Lut). Our results demonstrate that dioxin inhibited ALP activity, migratory capacity, and matrix mineralization, whereas co-treatment with each of the antagonists mitigated these effects. Dioxin also inhibited BMSC chemotaxis, while co-treatment with several antagonists partially rescued this effect. RNA and protein expression studies found that dioxin down-regulated numerous pro-osteogenic targets, whereas co-treatment with Ahr antagonists prevented these dioxin-induced expression changes to varying degrees. Our results suggest that dioxin adversely affects bone regeneration in a myriad of ways, many of which appear to be mediated by the Ahr. Our work suggests that the Ahr should be investigated as a therapeutic target to combat the adverse effects of CS on bone healing.



## 1. Introduction

The impact of tobacco smoke on human health remains a critical problem worldwide. Cigarette smoke (CS) has a well-established role in the pathogenesis of numerous smoking-related disorders, including chronic obstructive pulmonary disease (COPD), cancer, and atherosclerosis (Middlekauff et al., 2014; Sasco et al., 2004). Although less frequently recognized, smoking also exacerbates musculoskeletal disease and presents serious challenges in the treatment of orthopaedic conditions (Porter and Hanley, 2001). In addition to promoting osteoporosis, degenerative disc disease, and wound complications, smoking drastically hinders osseointegration and bony union - deleterious outcomes that are associated with higher rates of revision procedures (Sloan et al., 2010; Schmitz et al., 1999). In spine surgery, smoking has been shown to negatively impact outcomes, with a pseudarthrosis rate nearly double that of non-smokers (26.5 vs. 14.2%) (Glassman et al., 2000). Although the adverse effects of smoking have been studied most extensively in spine research, similar effects are seen in other orthopaedic conditions as well, especially tibial fracture healing (Patel et al., 2013). Currently, surgeons are limited in their ability to treat these patients, and are left with the difficult choice of refusing surgical intervention or performing procedures with significantly increased risks.

Determining a singular mechanism by which CS inhibits bone growth is problematic, as smoke contains upwards of 4000 distinct chemical constituents (Hoffmann and Hoffmann, 1997; Castillo et al., 2005). However, several mechanisms are understood to be involved. Nicotine is a potent anti-inflammatory and immunosuppressive, and has been shown to have deleterious effects on fibroblasts, red blood cells, and macrophages (Zevin et al., 1998; Jorgensen et al., 1998; Leow and Maibach, 1998), in addition to diminishing blood flow to tissues by promoting vasoconstriction (Leow and Maibach, 1998; Bornmyr and Svensson, 1991). Interestingly, the overall impact of nicotine on bone formation is still uncertain, and may be concentration-dependent; high concentrations of nicotine have been shown to inhibit osteoblast proliferation, whereas low concentrations actually have a proliferative effect (Rothem et al., 2009; Daffner et al., 2015;

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Gotfredsen et al., 2009; Syversen et al., 1999). Numerous studies have proposed that reactive oxygen species and other pro-inflammatory constituents and metabolites are responsible for dysregulation of bone homeostasis, reduction in bone mineral density, and inhibition of fracture healing (Rothem et al., 2009; Syversen et al., 1999; Holzer et al., 2012).

Recent work has implicated the Aryl Hydrocarbon Receptor (Ahr) as a mediator of anti-osteogenic effects. The receptor binds an extensive array of exogenous ligands, such as natural plant flavonoids, polyphenolics, and indoles, as well as xenobiotic toxicants, such as polycyclic aromatic hydrocarbons (PAH, e.g. benzo[*a*]pyrene), halogenated aromatic hydrocarbons (HAH, e.g. dioxins), and polychlorinated biphenyls (PCBs). PAHs and similar compounds are formed during the incomplete combustion of organic matter, including tobacco (Leow and Maibach, 1998). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) is a halogenated aromatic hydrocarbon with incredibly high-affinity for the Ahr. As such, dioxin is a commonly used probe to investigate the role of the receptor on various biological systems and endpoints (Ryan et al., 2007). More recently, Ahr activation by dioxin has been shown to have significant adverse effects on bone (Singh et al., 2000). For example, downstream effects resulting from Ahr activation have been shown to inhibit osteoblast function and differentiation, resulting in reduced ossification (Jamsa et al., 2001; Naruse et al., 2002; Ryan et al., 2007).

In previous work, we found that chronic exposure to dioxin inhibits BMP-2-mediated bone regeneration and posterolateral (L4-L5) spine fusion in rats (Hsu et al., 2015). Cessation of exposure for a period of 4 half-lives facilitated a partial recovery of regenerative capacity. These pre-clinical findings further supported previous work that identified bone as a sensitive target for dioxin, and suggest a potential link between ligand-induced Ahr activation and the reduced healing rates seen in smokers after spinal arthrodesis. However, the mechanisms of dioxin action on the bone regenerative process are still unclear. With this study, we sought to clarify these mechanisms and identify a viable therapeutic strategy to mitigate these effects. Numerous Ahr antagonists of both synthetic and natural origin have shown the potential to protect against the adverse effects of dioxin and other exogenous Ahr ligands for various biological endpoints (Dong et al., 2010). We hypothesize here that the use of one or more of these compounds to limit Ahr activation could reduce the adverse effects of dioxin on osteogenic differentiation and bone healing.

## 2. Materials and Methods

### 2.1. BMSC isolation and culture

Bone marrow stromal cells (BMSC) were harvested from femurs and tibiae of six-week-old female Long-Evans rats purchased from Charles River Laboratories (Chicago, IL). Animals were euthanized under anesthesia in accordance with Institutional Animal Care and Use Committee (IACUC)-approved procedures, and animals were housed under controlled temperature (23 ± 1 °C) and relative humidity (50 to 60%). Isolated BMSC were incubated with standard media comprised of Dulbecco's Modified Eagle Medium (DMEM; Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 20 mM HEPES sodium salt, 50 μg/mL streptomycin, and 50 μg/mL gentamycin sulfate. After 3–5 days of incubation (at 80% confluence), cells were re-plated and grown in either standard media (SM) or osteogenic media (OM; comprised of standard media supplemented with 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate, and 10 nM dexamethasone). Cells were treated with either vehicle control (dimethyl sulfoxide, DMSO; 0.1% final concentration) or the following: 10 nM dioxin, 50 μM nicotine, 0.5 μM α-Naphthoflavone (ANF), 4 μM resveratrol (Res), 10 μM 3,3′-Diindolylmethane (DIM), 0.2 μM luteolin (Lut), or dioxin + ANF, Res, DIM or Lut. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Treatment media was replaced twice per week at a minimum.

### 2.2. Alkaline Phosphatase activity

Alkaline Phosphatase (ALP) activity was quantitated using the SensoLyte pNPP Alkaline Phosphate Assay kit (Anaspec, Fremont, CA) and normalized to total protein. After supernatants were collected, enzymatic reactions were performed according to manufacturer's instructions. A minimum of three independent experiments were performed for quantitation of ALP activity as well as all other in vitro assays.

### 2.3. Matrix mineralization

BMSC were inoculated into 6-well plates at $1 \times 10^4$ cells per well. Cells were maintained in either standard or osteogenic growth conditions for 2 weeks, and were re-treated every 2–3 days. After 2 weeks, live cells were quantitated using an MTS assay (Promega, Madison, WI) for normalization purposes. Adherent cells were then washed twice with PBS, fixed with 4% paraformaldehyde, and stained with 2% Alizarin red solution. After collection of digital images, cells were destained with cetylpyridinium chloride, and $A_{540}$ was quantified using a Cytation 3 spectrophotometer (BioTek Instruments, Winooski, VT).

### 2.4. Cell migration

The effect of dioxin on dermal wound closure was assessed using the CytoSelect Wound Healing Assay Kit (Cell Biolabs Inc., San Diego, CA). When BMSC cells reached confluence, the inserts were removed from the wells and washed twice with PBS. Cells were then incubated in standard media containing DMSO or dioxin for 15 h, after which time wells were stained according to the manufacturer's instructions. Representative digital images were collected at time points of 0, 8, 15, and 24 h with a light microscope in order to evaluate the rate of "wound" closure. The migration distance across each wound was quantified by a comparison of final and initial wound widths followed by calculation of the percent change.

### 2.5. Chemotaxis

Pre-treated cells were trypsinized and counted using a Countess automated cell counter (Invitrogen, Grand Island, NY). $2 \times 10^5$ cells were suspended in 100 μL of migration buffer (standard media containing 0.2% FBS/0.1% bovine serum albumin) and inoculated into the upper chambers of 24-well transwell inserts (8 μm pore size). The lower chambers were inoculated with 400 μL of migration buffer supplemented with one of the following: 200 ng/mL CXCL12, 200 ng/mL IL-8, 200 ng/mL CCL20, or 200 ng/mL BMP-2. Wells containing only migration buffer in both the upper and lower chambers were included as negative controls. Membranes were then fixed with 4% paraformaldehyde and stained with 0.05% crystal violet. After removing cells from upper side using cotton applicators, cells adhered to the underside of the membrane were visualized and counted under a microscope by three independent observers, and an average cell count was computed for each treatment group.

### 2.6. RNA isolation and gene expression

Quantitative real-time polymerase chain reaction (QPCR) was performed on BMSC treated under osteogenic conditions with either DMSO or dioxin. After pre-treatment, mRNA was isolated from BMSC and expression levels were quantified and normalized to *Glyceraldehyde 3-phosphate Dehydrogenase* (*Gapdh*). Primer set was synthesized by Integrated DNA Technologies (Coralville, IA), with sequences detailed in Table 1. cDNAs were synthesized using a qScript cDNA Synthesis Kit (Quanta Bioscience, Gaithersburg, MD), and QPCR reactions were prepared with IQ SYBR Green Supermix (BioRad, Hercules, CA). QPCR was performed in the Equipment Core Facility of the Simpson Querrey Institute at Northwestern University using the following program: