# EXAMINATION OF MANDIBULAR AND MAXILLARY SQUAMOUS EPITHELIAL PROLIFERATION INDUCED IN MINK (*MUSTELA VISON*) BY 3,3',4,4',5-PENTACHLOROBIPHENYL (PCB 126)

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ABSTRACT

EXAMINATION OF MANDIBULAR AND MAXILLARY SQUAMOUS EPITHELIAL PROLIFERATION INDUCED IN MINK (*MUSTELA VISON*) BY 3,3',4,4',5-PENTACHLOROBIPHENYL (PCB 126)

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Mink (*Mustela vison*) exposed to 2,3,7,8,-tetrachlorodibenzodioxin (TCDD) and other TCDD-like compounds, such as 3,3',4,4',5-pentachlorobiphenyl (PCB 126), develop an invasive jaw lesion characterized histologically as mandibular and maxillary squamous cell proliferation. Jaw lesions have been reported in wild mink residing in environments contaminated with TCDD-like chemicals and there is evidence suggesting jaw-related lesions in other wildlife species exposed to TCDD-like chemicals. To date, no studies have been published addressing the etiology of this lesion, but the aryl hydrocarbon receptor (AhR) is assumed to be involved because of the lesion's induction by TCDD-like chemicals. To further examine the development of the lesion, two studies were conducted. The first study was a timeline study of the development of the lesion over a period of 28 days in mink exposed to 30 µg PCB 126/kg feed. In the second study, resveratrol, an AhR antagonist, was administered (6 daily gavage doses of 50 mg resveratrol/kg bw) to mink dosed with PCB 126 (a single IP injection of 30 µg PCB 126/kg bw following the last dose of resveratrol) to determine if it could prevent or reduce the development of the lesion. Resveratrol had no apparent effect on the incidence or severity of the PCB 126-induced effects

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KEY TO SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| AhR | Aryl hydrocarbon receptor |
| AHH | Aryl hydrocarbon hydroxylase |
| BMD | Bone mineral density |
| BSA | Bovine serum albumin |
| CHS | Chediak-Higashi syndrome |
| EDTA | Ethylenediaminetetraacetic acid |
| EROD | Ethoxyresorufin-*O*-deethylase |
| CYP1A1 | Cytochrome P450, family 1, member A1 |
| PBBs | Polybrominated biphenyls |
| PCBs | Polychlorinated biphenyls |
| PCDDs | Polychlorinated dibenzo-*p*-dioxins |
| PCDFs | Polychlorinated dibenzofurans |
| PCB 126 | 3,3',4,4',5-Pentachlorobiphenyl |
| PHAHs | Polyhalogenated aromatic hydrocarbons |
| TCDD | 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin |
| TEF | Toxic equivalency factor |
| TEQ | Toxic equivalents |

# CHAPTER 1

# LITERATURE REVIEW

## Polyhalogenated aromatic hydrocarbons

Polyhalogenated aromatic hydrocarbons (PHAHs) are a group of chemicals that are hydrophobic, resistant to metabolism, and ubiquitous in the environment. Their resistance to metabolism and their lipophilicity allows them to bioaccumulate and biomagnify within food chains. The general class of PHAHs includes chlorinated insecticides such as DDT and its metabolites, as well as polybrominated diphenyl ethers (PBDEs), bisphenol A (BPA), polybrominated biphenyls (PBBs), polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs), and polychlorinated dibenzo-p-dioxins (PCDDs). The principal PHAHs of interest here include the PCDDs, PCDFs and PCBs. Of these polychlorinated hydrocarbons (PCHs), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is considered to be the most toxic due to its coplanar structure (Figure 1.1) and its affinity for the aryl hydrocarbon receptor (AhR). Chemicals that are similar in structure to TCDD also bind to the AhR and induce a similar suite of toxic effects. These TCDD-like compounds include seven PCDD congeners, 10 PCDF congeners, and 12 PCB congeners. Of the coplanar PCB congeners, 3,3',4,4',5-pentachlorobiphenyl (PCB 126) is considered to be the most toxic (Bursian 2007).

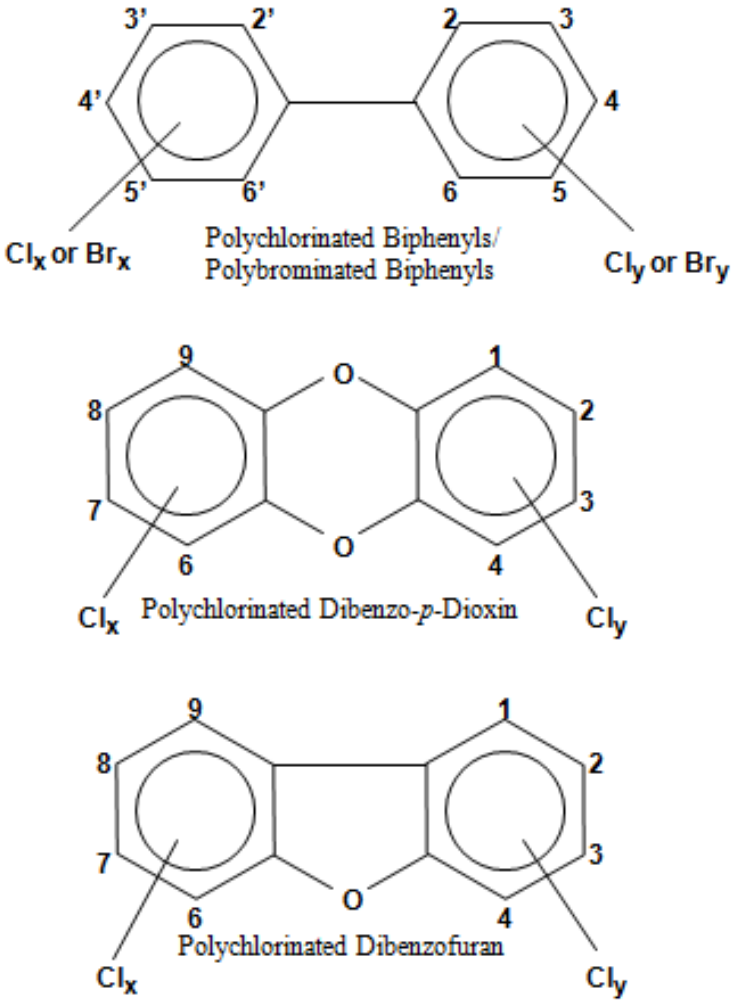



Figure 1.1 Chemical structures of PCBs, PCDDs, and PCDFs (Bursian 2007).

Table 1.1 PCB congeners with their IUPAC numbers and World Health Organization toxic equivalency factors for mammals, adapted from Van den Berg et al. (1998).

| **IUPAC number** | **PCB congener** | **WHO TEF** |
|---|---|---|
| 77 | 3,3',4,4'-TetraCB | 0.0001 |
| 81 | 3,4,4',5-TetraCB | 0.0001 |
| 126 | 3,3',4,4',5-PentaCB | 0.1 |
| 169 | 3,3',4,4',5,5'-HexaCB | 0.01 |
| 105 | 2,3,3',4,4'-PentaCB | 0.0001 |
| 114 | 2,3,4,4',5-PentaCB | 0.0005 |
| 118 | 2,3',4,4',5-PentaCB | 0.0001 |
| 123 | 2',3,4,4',5-PentaCB | 0.0001 |
| 156 | 2,3,3',4,4',5-HexaCB | 0.0005 |
| 157 | 2,3,3',4,4',5'-HexaCB | 0.0005 |
| 167 | 2,3',4,4',5,5'-HexaCB | 0.00001 |
| 189 | 2,3,3',4,4',5,5'-HeptaCB | 0.0001 |

The basic structures of TCDD-like compounds are very similar. These chemicals are composed of two benzene rings linked together by either a single bond between carbon atoms or the carbons are linked through an oxygen atom. The other carbons of the benzene rings can then form single bonds to halogen atoms such as chlorine or bromine (polybrominated biphenyls are also toxicants of concern)(Safe 1990).

Because of the structural similarity of these compounds, TCDD-like chemicals have the same mechanism of action, resulting in similar clinical signs of toxicity. In addition, the TCDD-like chemicals often occur together in complex mixtures throughout the environment. One approach to quantifying the total exposure to TCDD-like chemicals in a complex mixture is the toxic equivalency factor or TEF approach. A TEF describes the relative potency of the TCDD-like chemical based on toxicity data, cytochrome P4501A1 induction (to be described in greater detail in a subsequent section) or the chemical's binding affinity to the AhR compared to TCDD. The TCDD-like toxicity, expressed as toxic equivalents (TEQs), associated with a specific TCDD-like chemical is the product of the chemical's concentration and its TEF value. The TEF of a compound can be species-specific and therefore has been assigned differently for mammals, fish, and birds. Table 1.1 exhibits the TEF values of PCB congeners for mammals. The total TCDD-like toxicity associated with a complex mixture is the sum of the TEQs contributed by each TCDD-like constituent, which can be explained by the following equation: TEQ = $\Sigma_{n1}[PCDD_i \times TEF_i] + \Sigma_{n2}[PCDF_i \times TEF_i] + \Sigma_{n3}[PCB_i \times TEF_i]$. The TEF approach is a valuable tool used for the purposes of risk assessment of complex mixtures of TCDD-like chemicals. TEQs are particularly useful when using biological tissues or other samples taken from the environment to predict potential risk of exposure (Van den Berg et al. 1998).

TCDD-like compounds originate from a variety of sources and can result from either intentional processes (such as industrial activities) or as by-products of other processes (such as combustion). Polychlorinated biphenyls, for example, were produced in the United States between 1929 and 1977 as commercial mixtures of congeners for industrial purposes (Erickson and Kaley 2011). Due to their stability, they were used as insulators in transformers, as well as in smaller electrical appliances. Other uses included their addition to plasticizers, paints, fire retardants, and carbonless copy paper. Monsanto was the main producer of commercial PCB mixtures in the United States, which were sold under the trade name of "Aroclor". Companies throughout Europe and Asia also produced commercial mixtures of PCBs and marketed them under a variety of trade names. In contrast, PCDDs and PCDFs are by-products of industrial processes and combustion reactions and were never commercially produced (Breivik et al. 2004).

PCBs with different numbers of chlorine atoms attached to different positions on the biphenyl rings are called "congeners". There are 209 congeners, or possible combinations of molecules, that can be produced by chlorination of the biphenyl ring(s). Based on the position of chlorines attached to the rings, a polychlorinated biphenyl can assume different configurations. In Figure 1.1, the 2, 2', 6, and 6' positions of the PCB molecule are referred to as the "ortho" positions, the 3, 3', 5 and 5' positions are referred to as the "meta" positions, and the 4 and 4' positions are called the "para" position. These different positions are significant for description of PCB congeners because they influence the congener's toxicity potential. If the PCB congener is chlorinated at one or more of the meta or para positions and not at the ortho positions, the two rings will lie in the same plane (non-ortho, coplanar). These congeners are the most toxic and their effects are mediated by the AhR. The phenyl rings of congeners with chlorine atoms attached to one or both ortho positions decrease rotation due to steric interactions between the

chlorine atom and the adjacent ring or between the chlorine atoms. This change in shape reduces the ability of the congener to bind to the AhR, thus reducing toxicity (Safe 1990).

**Aryl hydrocarbon receptor**

The presumed mechanism of action of TCDD-like chemicals is through their action as AhR ligands. The AhR is an intracellular receptor that regulates gene transcription when activated by a ligand. Congeners of PCDDs, PCDFs, and PCBs that are co-planar, and thus resemble TCDD in structure, are potent xenobiotics that bind to the AhR. Once a ligand binds to the AhR, the ligand-receptor complex undergoes conformational change, which exposes the nuclear localization sequence. The ligand-receptor complex translocates into the nucleus, and activates transcription of several genes including cytochrome P450 1A1 (CYP1A1) (Denison and Nagy 2003). Cytochrome P450 enzymes comprise a family of mixed function oxidase enzymes that metabolize xenobiotics. The subfamily1A is located primarily in the endoplasmic reticulum of liver cells, and has been studied extensively for its role in metabolizing PHAHs (Sheweita 2000). When the AhR ligand is a xenobiotic, the gene expression is inappropriate and can cause deleterious effects in the organism (Denison et al. 2002). Effects of TCDD-like compounds attributed to interaction with the AhR include: reproductive toxicity, neurotoxicity, immunotoxicity, tumor promotion, and genetic polymorphism (Mandal 2005).

There are still many questions about the function of the AhR, but it is considered to be the primary route by which TCDD-like chemicals act, as experiments with AhR-null mice have shown (Okey 2007). An AhR null mouse is genetically modified so that it does not express the AhR receptor protein, and the target genes of the AhR are not induced upon exposure to TCDD. The AhR-null mice expressed abnormalities including deficiency of the immune system,

especially in very young or very old animals. Additionally, effects such as lung and liver tumors, calcifications in the uterus, proliferation of skin cells, and heart hypertrophy were described in AhR-null mice. It has also been observed that AhR-null mice have decreased fertility, which suggests that the AhR plays a role in reproductive function. Additionally, there is evidence that the AhR can have a role in the inflammatory and immune response (Kawajiri and Fujii-Kuriyama 2007). These effects have implications for the AhR's natural role in cell and tissue development, and in homeostasis. However, the importance of these studies in regard to TCDD toxicity, is that these mice showed none of the deleterious effects associated with exposure to TCDD such as wasting syndrome, lipid accumulation in liver cells, atrophy of the thymus, and cleft palate, even at doses as high as 2000 µg/kg body weight. This indicates that the AhR has a significant role in mediating many TCDD-related effects in mammals (Gonzalez and Fernandez-Salguero 1998).

The AhR also has presumed natural ligands, which may be dietary in source. These natural ligands tend to be weak ligands that degrade quickly after having induced gene expression (Denison et al. 2002). Comparatively, PHAHs are very persistent in the organism as well as in the environment. Due to the fact that persistent expression of the AhR is needed to cause deleterious effects in organisms, metabolically stable ligands are the most potent (Denison and Nagy 2003), which is one reason why PCB 126 is of one of the most toxic PCHs.

Decreases in bone mineral density and altered bone development in babies are among the effects that have been well documented as problems associated with exposure to TCDD-like compounds and they are of particular importance to the present study. These effects will be discussed in further detail in the next section; however, it is important to note the potential role that the AhR plays in these effects. Two types of cells, osteoblasts and osteoclasts, which arise

from stem cells, are important not only to bone formation, but also to bone maintenance. Bone tissue is constantly being formed by osteoblasts and resorbed by osteoclasts, which is called bone remodeling. If this equilibrium is upset, then bone disease such as osteoporosis could occur (Ducy et al. 2000). Recent studies have shown that the AhR plays a role in osteoblast and osteoclast differentiation from stem cells *in vitro* (Ryan et al. 2007; Korkalainen et al. 2009), which could explain the correlation seen between bone-related effects and exposure to TCDD-like chemicals in many species of mammals. Korkalainen et al. (2009) showed that TCDD can inhibit osteoblast differentiation *in vitro*. In a related study, two strains of rats with a difference in sensitivity to TCDD due to differences in AhR structure were compared for differences in tibial bone growth. The more sensitive strain had significantly reduced bone growth after exposure to TCDD compared to the less sensitive strain, which indicated that the AhR plays a role in bone development (Jamsa et al. 2001).

**Effects of TCDD and TCDD-like compounds**

TCDD-induced effects are expressed as a variety of endpoints including reproductive failure, teratogenesis, wasting syndrome, hepatic toxicity, carcinogenicity in various tissues, and death. Chloracne is a major effect experienced by humans exposed to high doses of TCDD-like compounds (Dobrzynski et al. 2009). The types and severity of toxic effects observed can be affected by animal species (or strain), age, and sex (Safe 1990).

On a biochemical level, TCDD-like compounds induce phase I metabolic enzymes, such as the cytochrome P-450 1A1 and 1A2 enzymes, which include aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin-O-deethylase (EROD) (Safe 1990). The measurement of EROD activity is a commonly used biomarker of TCDD-like chemical exposure (Kafafi et al. 1993), which has been studied extensively in fish (Whyte et al. 2000), but has more recently been used

in mammalian models, such as mink (*Mustela vison*) (Moore et al. 2009). EROD activity has been measured in many tissues including liver, kidney, brain and gastrointestinal tract cells and can therefore be used to evaluate biologically active TCDD-like compounds in a variety of tissues.

Another effect of TCDD-like compounds including PCBs, is the decrease in bone mineral density (BMD) associated with exposure of mammalian wildlife species to these types of chemicals. A study conducted at a former military site contaminated with PCBs in Labrador, Newfoundland, Canada demonstrated that BMD of resident deer mice was decreased (Johnson et al. 2009). A study that evaluated the change in BMD in Baltic grey seals over the past century in relation to the increase in environmental contamination with organochlorine chemicals showed a strong correlation between a loss in bone density and an increase in skull bone lesions with increasing environmental concentrations of organochlorine contaminants (Lind et al. 2003).

Not all studies, however, have indicated that a change in BMD is attributable to exposure to organochlorine chemicals. A study in which male arctic foxes were fed PCB-contaminated minke whale blubber failed to show a significant difference in bone density when these animals were compared to the control group, which was fed pork fat (Sonne et al. 2009). The same authors examined the relationship of polar bear skull pathology to concentrations of organochlorine contaminants, but found no strong correlation between the two factors (Sonne et al. 2007).

In humans, the effects of TCDD-like chemicals range from chloracne to developmental tooth disorders, as reported in studies of people affected by the 1979 Seveso disaster in Italy, in which an explosion caused the release of TCDD over an area of 18 $km^2$. Studies of this incident have shown that tooth development disorders were found in children 25 years later as a result of

this exposure (Alaluusua and Lukinmaa 2006). Further studies have shown that TCDD affects tooth development in many animal species (Dobrzynski et al. 2009). Two other contamination incidents resulted in the exposure of populations to PCB-contaminated rice oil. The "Yusho" incident occurred in Japan in 1968, and the "Yu-cheng" incident was a similar exposure event that occurred in Taiwan in 1979. In both of these cases, cooking oil was contaminated with PCBs and was ingested by many people before the problem was discovered. Over 3,000 people were victims of these incidents, and they suffered from a variety of deleterious effects including chloracne and a variety of dental problems. It was shown that children of women who were pregnant at the time of the Yu-cheng incident have experienced developmental defects of the teeth and gums (Wang et al. 2003). Several studies have reported dose-response relationships between PCB concentrations in the serum of women exposed during the Yu-cheng incident and effects such as missing teeth, microdontia, pigmentation, enamel hypoplasia, and impaction in the offspring of those women (Alaluusua and Lukinmaa 2006).

In addition to these incidents of human exposure, children in Slovenia who lived near a contaminated agricultural area experienced conditions such as demarcated opacities and hypoplasia in teeth and children of factory workers in various parts of the world who were exposed to PCBs occupationally exhibited clinical signs such as gingival pigmentation, mottled enamel, and dental caries in primary teeth (Alaluusua and Lukinmaa 2006).

The effects of TCDD-like compounds on tooth development may be dependent both on the dose and the stage of development (or status of bone-remodeling) during which exposure occurs. For example, exposure at very early stages can prevent full tooth formation while exposure at later stages can affect the differentiation of ameloblasts and odontoblasts, which can affect the formation of enamel and dentin. Because human tooth development begins in the

fourth month of pregnancy and continues through the first year of life, exposure to TCDD-like compounds during nursing is of concern. It has been reported that exposure of adult rats to TCDD caused death of odontoblasts and skull demineralization. Alaluusua and Lukinmaa (2006) suggest that the AhR may be indirectly related to these effects by signaling the epidermal growth factor receptor, a cascade potentially involved in tooth development.

TCDD-like compounds have also been considered to be carcinogenic in some animals. While they are not complete carcinogens, they have been shown to play a role in tumor promotions, especially of hepatic cancers that have been initiated by a complete carcinogen (Knerr 2006). Furthermore, Yoshizawa et al. (2005) conducted studies which exposed Harlan Sprague-Dawley rats to dioxin-like compounds via gavage and induced what they considered squamous cell carcinomas in the oral cavity.

**Aryl hydrocarbon receptor antagonism**

Several compounds have been shown to antagonize the effects of TCDD-like compounds, presumably by binding to the AhR without inducing gene expression and therefore causing the expected deleterious effect (Denison et al. 2002). In vitro screening assays show that many flavanoids, especially flavones and flavonols, and phenolic compounds such as resveratrol and curcumin found in dietary sources exhibit such antagonistic activity (Amakura et al. 2003).

Both *in vitro* and *in vivo* studies have shown resveratrol (3,5,4'-trihydroxystilbene) to have strong antagonistic properties without inducing toxicity of its own. It has been confirmed that resveratrol binds to the AhR, and blocks TCDD induction of the CYP1A1 protein, thereby preventing mediation of the deleterious effects associated with TCDD toxicity. The main dietary source of resveratrol is red wine (Casper et al. 1999). Of particular interest is a study by Singh et al. (2000) that examined the ability of resveratrol to inhibit the effects of TCDD on bone

formation. In an *in vitro* study that tested two bone-forming models, the bone formed by cells treated with TCDD and resveratrol was comparable to the bone formed by cells treated with resveratrol alone and control cells, whereas cells treated with TCDD formed little bone. The authors described what "appeared to be a complete reversal of the TCDD effect in cultures treated with both TCDD and resveratrol" as an indication of successful AhR inhibition (Singh et al. 2000). *In vivo* studies have shown that resveratrol can lessen the teratogenic effects of TCDD such as cleft palate (Jang et al. 2008).

**Mandibular and maxillary squamous epithelal proliferation in mink**

One specific effect induced by TCDD and PCB 126 that has generated interest among federal regulatory agencies is a jaw lesion in mink. Histologically, this lesion has been characterized as mandibular and maxillary squamous epithelial proliferation that form nests and cords adjacent to the teeth. These squamous cells invade the adjacent alveolar bone leading to severe osteoporosis. Gross observations reveal red, swollen gums and bleeding around the teeth, as well as crooked, displaced and lost teeth (Render et al. 2000a,b, 2001). It has been hypothesized that the development of this lesion is mediated by the AhR. The lesion could be caused by chemical-induced stimulation of the epithelial cells in the rests of Malassez, which produces a bone-resorbing factor (Render et al. 2000b); however, there have been no studies that have addressed this hypothesis.

Of particular interest is the fact that this lesion has been seen at concentrations of PCB 126 that are less than those that result in reproductive effects (Beckett 2005), and that they have been induced by environmentally relevant concentrations of TCDD-like contaminants. The lesion was induced in ranch mink that were fed diets containing fish collected from rivers around the US including the Saginaw River in Michigan, and the Housatonic River in Massachusetts

(Bursian et al. 2006a,b). These studies indicate that the environmental concentrations of TCDD-like compounds are cause for concern for piscivorous wildlife in those environments. Field studies have indicated that this jaw lesion is also seen in wild mink. In a field study of the Kalamazoo River in Michigan, it was shown that the lesion was present in wild mink trapped in the vicinity of the river. The Kalamazoo River has been designated as a Superfund site due to its PCB contamination, which was caused by waste from the processing and recycling of carbonless copy paper (Beckett et al. 2005). The most recent study reported that mink on the south shore of Lake Ontario, which is contaminated with PCBs, exhibited the lesion. This study was also the first time that a captured mink had gross and histological signs of the lesion (Haynes et al. 2009). More study of this lesion is needed in order to better understand the relationship between TCDD-like chemicals and the lesion, how the lesion develops, and the ecological implications of the lesion.

**Jaw related effects in other species**

Gross lesions similar to those induced by TCDD-like chemicals in mink have been described in marine mammals such as Baltic grey seals (Bergman et al. 1992), as well as terrestrial mammals such as rats (Yoshizawa et al. 2005), and raccoons (Hungerford et al. 1999), although a cause-effect relationship has not been firmly established for these species (Render et al. 2000a). One study reported a strong correlation between the prevalence of skull bone lesions in harbor seals in the Baltic region and the increase in persistent organic pollutants over time in the environment (Mortensen et al. 1992). The lesion in harbor seals was described as "considerable loss of alveolar bone around teeth and other parts of the jaw, as well as multiple loss of teeth", which is very similar to the description of the gross jaw lesion in mink (Render et al. 2001). Similarly, studies on beluga whales found dead in the St. Lawrence Estuary between

1982 and 1991 reported tooth loss in addition to periodontitis (Beland et al. 1993; De Guise et al. 1995). However, in a related study to that by Beland et al. (1993), it was noted that there were surprisingly low concentrations of PCDDs and coplanar PCBs found in the tissues, suggesting that these animals may metabolize TCDD-like compounds, and that a metabolite could be the cause of the pathology (Norstrom et al. 1992). Similar results were noted in studies that examined skull pathology in polar bears collected from East Greenland and Svalbard. Displacement of teeth, periodontitis, osseous proliferations, and severe tooth wear were all described in the polar bears, but it was determined that there was no correlation between these effects and exposure to organochlorines (Sonne et al. 2007).

The effects of TCDD on jaw development in zebrafish (*Danio rerio*) have been studied extensively in different laboratories. The advantages of using this organism as a model are that they develop rapidly, and they have a transparent body, which aids in the observation of internal organ and skeletal development. Zebrafish are especially advantageous as a toxicological model because development of the embryos is affected by TCDD. Furthermore, the AhR has been characterized in this species, and more importantly, has been detected in the jaw cartilage of developing embryos exposed to TCDD (Teraoka et al. 2002). TCDD exposure during development has been shown to disrupt lower jaw cartilage growth (Teraoka et al. 2002). The mechanism for this disruption has been evaluated by Prasch et al. (2003), who showed that TCDD inhibits cartilage growth of the lower jaw in zebrafish embryos, but does not affect the individual components of the cartilage. This knowledge, however, may not be easily translated to mammalian jaw development, because zebrafish have two forms of the AhR, known as zfAHR1 and zfAHR2. While zfAHR1 is the form most similar to that found in mammals, it is zfAHR2 that mediates the jaw effects (Prasch et al. 2003). Xiong et al. (2008) assessed

expression of genes mediated by AhR in zebrafish, showing that there is a reduction in the expression of *sox9b* by exposure to TCDD, resulting in some types of jaw malformations. These results could explain partially the mechanism by which TCDD affects jaw formation in other organisms as well, if steps involved in jaw formation are similar. In a more recent study, Planchart and Mattingly (2010) propose that the gene *FoxQ1b* may also be involved in jaw malformations in developing zebrafish.

**Jaw related effects not associated with exposure to TCDD-like chemicals**

The blue iris color strain of mink naturally exhibit severe, destructive periodontal disease, which results in the loss of teeth (Hammer et al. 2005). It is assumed that the periodontal disease is due to immunosuppression as a result of inbreeding for this mink strain's pelt color. The gross clinical signs reported for iris mink are similar to those described in mink that have been exposed to TCDD-like chemicals. Thus, the possibility exists that the lesion occuring in mink exposed to TCDD-like chemicals is identical to that reported for blue iris mink, which would imply that the lesion results from immunosupression induced by any number of stressors including exposure to TCDD-like chemicals. A comparison of these lesions histologically could indicate whether this is the case.

**Mink as an animal model**

The mink is an ideal animal to use for modeling the jaw lesion well as deleterious effects that are indicative of exposure to TCDD-like chemicals. This is due to the fact that mink meet the criteria for a good sentinel species. They are sensitive to PCBs and other related environmental contaminants and mink are exposed to these contaminants in their natural environment. Mink are piscivorous and are at the top of the food chain, which allows them to

bioaccumulate pollutants such as TCDD-like compounds. Finally, they are maintained in captivity, which has allowed development of a robust biological database. Mink have been used in a variety of toxicology studies, and therefore are a good animal model that can be used in both laboratory and field studies (Basu et al. 2007).

CHAPTER 2

# TIMELINE STUDY OF THE DEVELOPMENT OF MANDIBULAR AND MAXILLARY SQUAMOUS EPITHELIAL PROLIFERATION IN MINK (*MUSTELA VISON*)

## INTRODUCTION

Polychlorinated biphenyls (PCBs) are a group of environmental contaminants that are found in the environment as a result of their past production for industrial uses. The physical properties of PCBs, which include lipophilicity and resistance to biochemical metabolism, result in environmental persistence and the ability to bioaccumulate and biomagnify. Twelve of the 209 individual PCB congeners are similar in structure to 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is considered to be the most toxic member of the polychlorinated hydrocarbons, and therefore act through the aryl hydrocarbon receptor (AhR) to cause a variety of well-documented toxic effects in wildlife, including reproductive and developmental toxicity, neurotoxicity, immunosuppression, tumor promotion, and genetic polymorphism (Mandal 2005). Of the 12 TCDD-like PCB congeners, 3,3',4,4',5-pentachlorobiphenyl (PCB 126) is most similar to TCDD in terms of structure as well as toxicity, and has therefore been assigned a TEF of 0.1 (Van den Berg et al. 1998). This congener is of particular concern in a number of areas contaminated with PCBs because it is primary contributor of TCDD-like toxicity (Bursian et al 2006a,b,c).

One particular effect of PCB 126 reported in mink (*Mustela vison*) is a jaw lesion characterized as mandibular and maxillary squamous cell proliferation that was first described by Render et al. (2001) as a proliferation of squamous cells arising out of the gingival tissue

adjacent to the teeth. These proliferations coalesce to form squamous cell cysts that invade the alveolar bone to cause osteoporosis, eventually causing loose, displaced teeth.

The occurrence of mandibular and maxillary squamous cell proliferation has recently been used to confirm environmental contamination at a number of locations, and therefore has the potential to be further developed as a biomarker for exposure to TCDD-like chemicals. The research on this topic has included two feeding studies in which mink were fed diets containing fish collected from the Saginaw River in Michigan (Bursian et al. 2006a) and the Housatonic River in Massachusetts (Bursian et al 2006b,c). The lesion was induced in mink at TCDD toxic equivalent (TEQ) concentrations of 47 and 9.2 ng TEQs/kg feed in the Saginaw River (Bursian et al., 2006a) and Housatonic River (Bursian et al., 2006b) studies, respectively. In addition to laboratory studies, wild mink trapped along a PCB-contaminated stretch of the Kalamazoo River in Michigan also exhibited histological signs of the lesion (Beckett et al. 2005) as did a mink collected from a PCB-contaminated site on the south shore of Lake Ontario (Haynes et al. 2009)

Despite numerous reports of the occurrence of the jaw lesion in mink exposed to TCDD-like chemicals, it is not known exactly when and where in the jaw tissue the proliferation begins to occur or the cellular changes that are involved in the lesion. The objectives of the present study was (1) to determine how quickly the jaw lesion begins to develop upon exposure to the inducing chemical, (2) identify the site where the actual proliferation begins and (3) the extent of lesion progression in the jaw tissue over time.

## METHODS

Ninety-six 8- to 12-week-old mink from the Michigan State University Experimental Fur Farm herd were randomly chosen from litters of six or more animals and assigned to one of 16 treatment groups. Each treatment group represented a specific number of days the animals were

to be given feed containing PCB 126 prior to necropsy. Necropsies were performed on the treated animals twice per week, on Tuesdays and Thursdays. Due to the fact that this was primarily a descriptive study, untreated control animals were not included in the experimental design. The study began on July 6, 2010 and ended on August 6, 2010 after only five weeks duration. Gross clinical signs, including loose teeth, were observed on the 24$^{th}$ day of the study leading to the decision to terminate the trial rather than complete the timeline assessment.

Mink were housed indoors, two mink per cage (61 x 80 x 47 cm), and provided feed and water ad libitum. The photoperiod was set to mimic natural conditions. Mink were fed a standard ranch diet containing PCB 126 (certified 99.7% pure, AccuStandard, New Haven, CT). To prepare the PCB 126 for incorporation into the mink feed, 2 ml hexane (EMD Chemicals, Gibbstown, NJ) was added to each of three vials containing 5 mg PCB 126 and the vials were shaken until the PCB 126 was dissolved. The solutions were transferred to an I-Chem jar (VWR International, Batavia, IL) containing 300 mL corn oil. An additional 1 ml hexane was added to each vial, shaken, and transferred to the corn oil. The corn oil mixture containing PCB 126 was then added to the liquid ingredients of the diet, which were mixed in a 400 kg capacity paddle mixer prior to adding the dry ingredients (Table 2.1). The total amount of PCB 126 added to the diet was 17.018 mg resulting in a calculated dietary concentration of 30 µg PCB 126/kg feed. Samples of the feed were taken throughout the mixing process and placed into two I-Chem jars, which were stored at -80$^{o}$C for subsequent chemical analysis. Feed was packed in 19-liter buckets and frozen at -7 °C. Individual buckets were thawed at 4 °C when needed for feeding. The same batch of feed was used throughout the study. Each animal was provided with approximately 250 g feed each day.

Table 2.1 Ingredients of diet (percent of total).

| Ingredient | % of diet |
|---|---|
| GNF 20 (cereal) | 18.3 |
| Chicken, whole ground | 23.8 |
| Liver, spray-dried | 2.7 |
| Eggs, spray-dried | 4.6 |
| Soy Oil | 5.0 |
| Water | 41.0 |
| Fish, dried ground | 2.7 |
| Vitamin premix | 0.43 |
| Mineral premix | 0.43 |
| Phosphoric acid | 1.0 |
| Biotin | 0.029 |
| Larvadex | 6.0 ml/100 lb |

Beginning on day 0, and then for days 3, 7, 10, 14, 17, 21, 24, and 28 days of PCB 126 exposure, six mink were euthanized with $CO_2$, and the thyroid and adrenal glands, kidneys, spleen, heart, liver, and brain were removed, weighed, and placed in 10% neutral buffered formalin. The jaws were removed, stripped of adhering skin and tissue and placed in 10% neutral buffered formalin (VWR International, West Chester, PA) for subsequent processing. Jaws then were placed in Rapid Decal (Leica Biosystems Peterborough, Peterborough, UK) for 36 to 48 hours for decalcification. After decalcification, jaws were trimmed, embedded in paraffin, sectioned (thickness, 5µm) and stained with hematoxylin and eosin. One section was taken for each set of jaw tissue, and the slides were examined under light microscope by a board-certified pathologist for signs of the jaw lesion. The lesions were graded on a scale of 0 to 3 (Table 2.2), as described in Beckett et al. (2005).

**Statistical Methods**

Organ masses were converted to percent of the body weight, subjected to arcsine transformation, and then analyzed for change over time. Due to the high variation in body size within and between treatment groups, it was determined to use relative organ weights rather than absolute. The Regression Procedure in SAS software (SAS: Statistical Analysis Software, version 9.2) was used to test for correlations between organ mass changes and time of exposure to the chemical (days), age of the animal (days), sex, and litter.

## RESULTS

**Relative Organ Mass**

Body weight was increasing through most of the study due to the age of the animals, because they were young and still growing. On day 28, however, compared to day 24 there was

a decrease in mean body weight. This can be attributed to the fact that by this time the animals were suffering from systemic toxic effects of the PCB 126 exposure. There was a significant correlation between duration of exposure to PCB 126 and an increase in relative mass only for the adrenal glands ($p = 0.0201$). There were no significant correlations between time of exposure and relative tissue mass for heart ($p=0.2389$), liver ($p=0.8027$), spleen ($p=0.8967$), brain ($p=0.1002$), kidneys ($p=0.3054$) and thyroid gland ($p=0.1846$). Tables 2.3 and 2.4 present mean relative organ mass at all time points.

**Pathology**

Figure 2.1 displays the number of mink with specific lesion scores on selected days of exposure to PCB 126. PCB 126–induced lesions were apparent on day zero, which may indicate a background incidence of these lesions. Moderate lesions were first seen on day 3 of exposure, and the incidence and severity of the jaw lesion increased with time of exposure. At 21 days of exposure, gross signs of the lesion were observed. Teeth, especially the bottom incisors, were displaced in many animals at necropsy. At 24 days of exposure, teeth were very loose, and in one animal the teeth began to fall out during necropsy.

Figure 2.2 is a photomicrograph of a mandible from an untreated mink to illustrate orientation of teeth and Figures 2.3 through 2.10 are photomicrographs that are representative of the progress of the lesion at each time point. The lesion first appeared as one or two squamous cell cysts that developed near the peridontal ligament. As the time of exposure increased, so did the number of cysts. In the slides of the earliest exposures, after three to seven days of exposure to PCB 126, there appeared only one or two squamous cell cysts in only one region of the mandible or maxilla of the animal. Clusters of cysts began to appear adjacent to the periodontal

ligament in a single region of the tissue (molar, premolar, or canine regions) of the mandible or maxilla, after seven to ten days of PCB 126 exposure. Severe lesions began to occur on days 10-14, which are described as groups of squamous cell cysts in more than one region of the jaw tissue. The severity of the lesion continued to progress until animals exposed for 21 through 28 days had squamous cell cysts completely covering the area surrounding the teeth. It was at this time that the teeth were loose enough to fall out during necropsy, indicating loss of alveolar bone. One male mink necropsied on day 28 showed no histological signs of the lesion.

Table 2.2 Description of jaw lesion histological scores, adapted from Beckett et al. (2005).

| Score | Lesion Description |
|---|---|
| 0 | Normal morphology, no squamous epithelial cell proliferation |
| 1 | Mild; focal (one or two) squamous epithelial cysts invading jaw bone, restricted to a single region of the dental arcade (molar, premolar, incisor) |
| 2 | Moderate; multiple squamous epithelial cysts (three or more), adjacent to several teeth or in two or more regions of the dental arcade |
| 3 | Severe; multiple squamous epithelial proliferations, larger in size or coalescing, cause significant bone infiltration and lysis and/or loss of teeth, proliferations may exhibit cellular atypia consistent with malignancy |

Table 2.3 Mean relative mass of heart, liver, spleen, and brain of mink exposed to PCB 126 for time periods ranging from 0 to 28 days.

| Days of | No. of | Body | Relative organ mass (as a percent of body mass)[1] | | | |
|---|---|---|---|---|---|---|
| exposure | animals | mass (g) | Heart | Liver | Spleen | Brain |
| 0 | 6 | 623±51 | 0.60 ± 0.066 | 5.2 ± 1.2 | 0.39 ± 0.10 | 1.85 ± 0.39 |
| 3 | 6 | 720±57 | 0.62 ± 0.047 | 5.4 ± 0.74 | 0.43 ± 0.094 | 1.45 ± 0.17 |
| 7 | 6 | 731±57 | 0.68 ± 0.039 | 5.4 ± 1.1 | 0.50 ± 0.20 | 1.44 ± 0.27 |
| 10 | 6 | 745±46 | 0.60 ± 0.048 | 5.8 ± 0.81 | 0.41 ± 0.086 | 1.33 ± 0.12 |
| 14 | 6 | 784±62 | 0.65 ± 0.073 | 5.2 ± 0.53 | 0.40 ± 0.064 | 1.40 ± 0.12 |
| 17 | 7 | 848±93 | 0.60 ± 0.079 | 5.8 ± 0.52 | 0.41 ± 0.12 | 1.31 ± 0.22 |
| 21 | 6 | 786±58 | 0.62 ± 0.092 | 5.5 ± 0.60 | 0.52 ± 0.16 | 1.30 ± 0.13 |
| 24 | 7 | 818±101 | 0.60 ± 0.055 | 6.6 ± 1.3 | 0.52 ± 0.16 | 1.31 ± 0.26 |
| 28 | 8 | 691±61 | 0.65 ± 0.057 | 6.2 ± 0.99 | 0.55 ± 0.16 | 1.56 ± 0.39 |

[1]Data presented as mean ± 95% confidence interval for relative organ mass and mean ±SE for body mass.

Table 2.4 Mean relative mass of kidneys, adrenal glands, and thyroid gland of mink exposed to PCB 126 for time periods ranging from 0 to 28 days.

| Days of exposure | No. of animals | Body mass (g) | Relative organ mass (as a percent of body mass)[1] | | |
|---|---|---|---|---|---|
| | | | Kidneys | Adrenal glands | Thyroid gland |
| 0 | 6 | 623±51 | 0.82 ± 0.15 | 0.0145 ± 0.0028 | 0.00522 ± 0.0012 |
| 3 | 6 | 720±57 | 0.79 ± 0.12 | 0.0102 ± 0.0016 | 0.00475 ± 0.00078 |
| 7 | 6 | 731±57 | 0.87 ± 0.24 | 0.0138 ± 0.0054 | 0.00507 ± 0.0016 |
| 10 | 6 | 745±46 | 0.87 ± 0.11 | 0.0128 ± 0.0028 | 0.00454 ± 0.00058 |
| 14 | 6 | 784±62 | 0.87 ± 0.063 | 0.0143 ± 0.0024 | 0.00495 ± 0.00073 |
| 17 | 7 | 848±93 | 0.95 ± 0.15 | 0.0170 ± 0.0069 | 0.00486 ± 0.0016 |
| 21 | 6 | 786±58 | 1.05 ± 0.39 | 0.0167 ± 0.0037 | 0.00467 ± 0.00083 |
| 24 | 7 | 818±101 | 0.97 ± 0.15 | 0.0220 ± 0.0095 | 0.00537 ± 0.0013 |
| 28 | 8 | 691±61 | 1.15 ± 0.37 | 0.0282 ± 0.018 | 0.00592 ± 0.0016 |

[1]Data presented as mean ± 95% confidence interval for relative organ mass and mean ±SE for body mass.

Figure 2.1 The effects of feeding mink diets containing PCB 126 on the severity of mandibular and maxillary squamous epithelial proliferation over time.

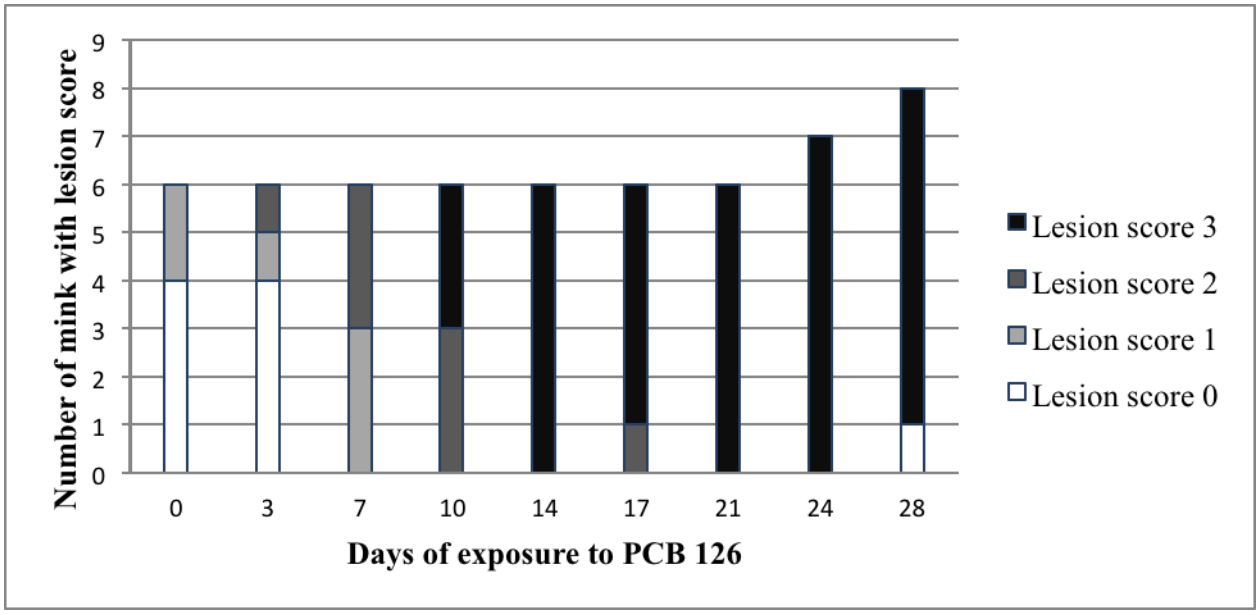


Figure 2.2 Mandible of an untreated mink (*Mustela vison)*. The three incisors are labeled $I_1$ through $I_3$, canines are labeled C, premolars are labeled $P_2$ through $P_4$, and molars are labeled $M_1$ and $M_2$ (Beckett 2005). For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

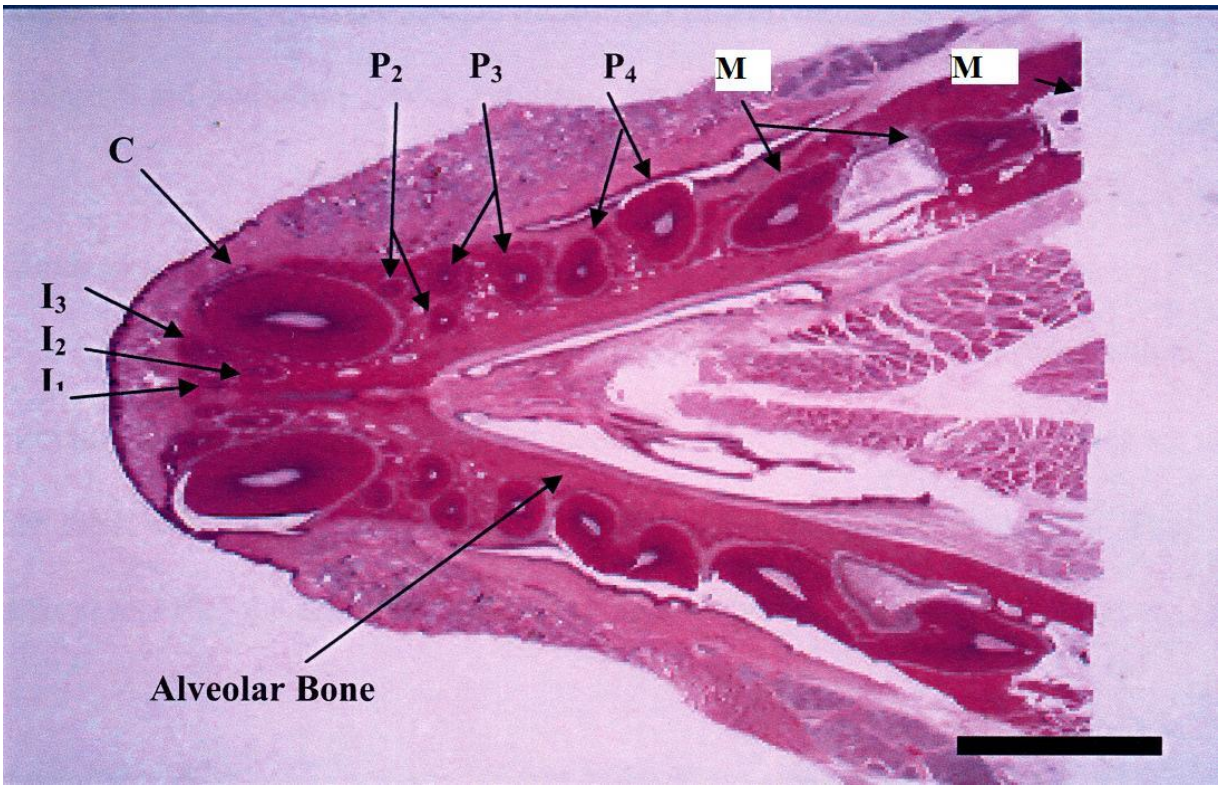


Figure 2.3 Photomicrograph of left mandibule (taken at magnification 4x) showing molars of a male mink collected on day 0, before exposure to PCB 126. T = tooth, AB = alveolar bone, PL = periodontal ligament. This is an example of normal jaw tissue (lesion score 0). Note the regularity of the tissues aurrounding the teeth.

Figure 2.4 Photomicrograph of left mandible (taken at magnification 4x) showing a molar of a female mink exposed to PCB 126 for 3 days. Arrows indicate squamous cell cysts, and the following letter codes define the tissues. T = tooth, AB = alveolar bone, PL = periodontal ligament, SCC = squamous cell cyst. This is an example of a mildly affected jaw (lesion score 1). Note the one squamous cell cyst (SCC) developing to the left of the molar (indicated by an arrow). The rest of the bone and periodontal ligament is healthy and there are no other morphological changes.

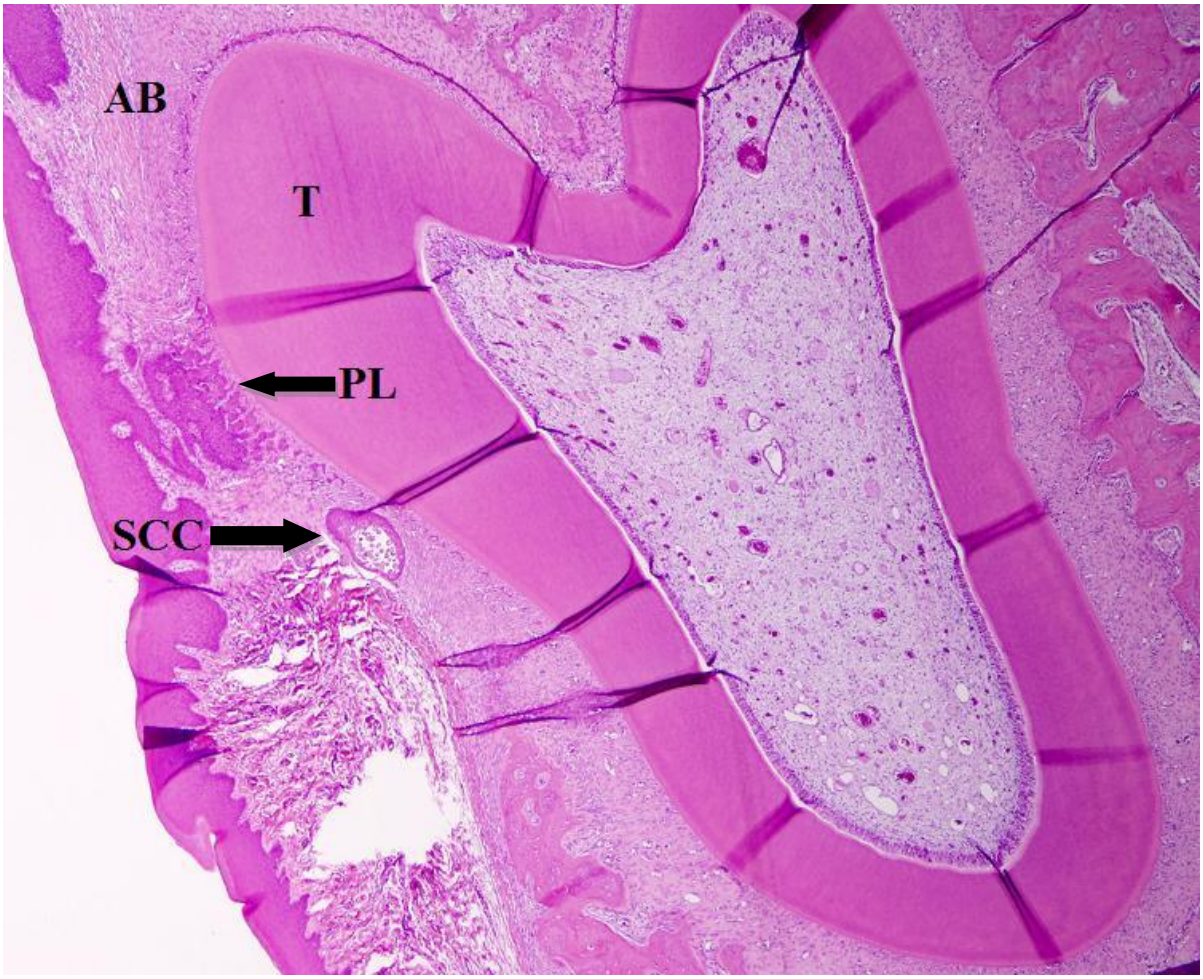


Figure 2.5 Photomicrograph of a right mandible (taken at magnification 4x) showing a molar of a male mink after 10 days of exposure. This is an example of a moderately affected jaw (lesion score 2). There is proliferation of nests of squamous cells (NSC) surrounding the periodontal ligament. There is one squamous cell cyst (SCC) separate from the tooth (indicated by arrow). Another arrow indicates a larger proliferation adjacent to the tooth (bottom left).

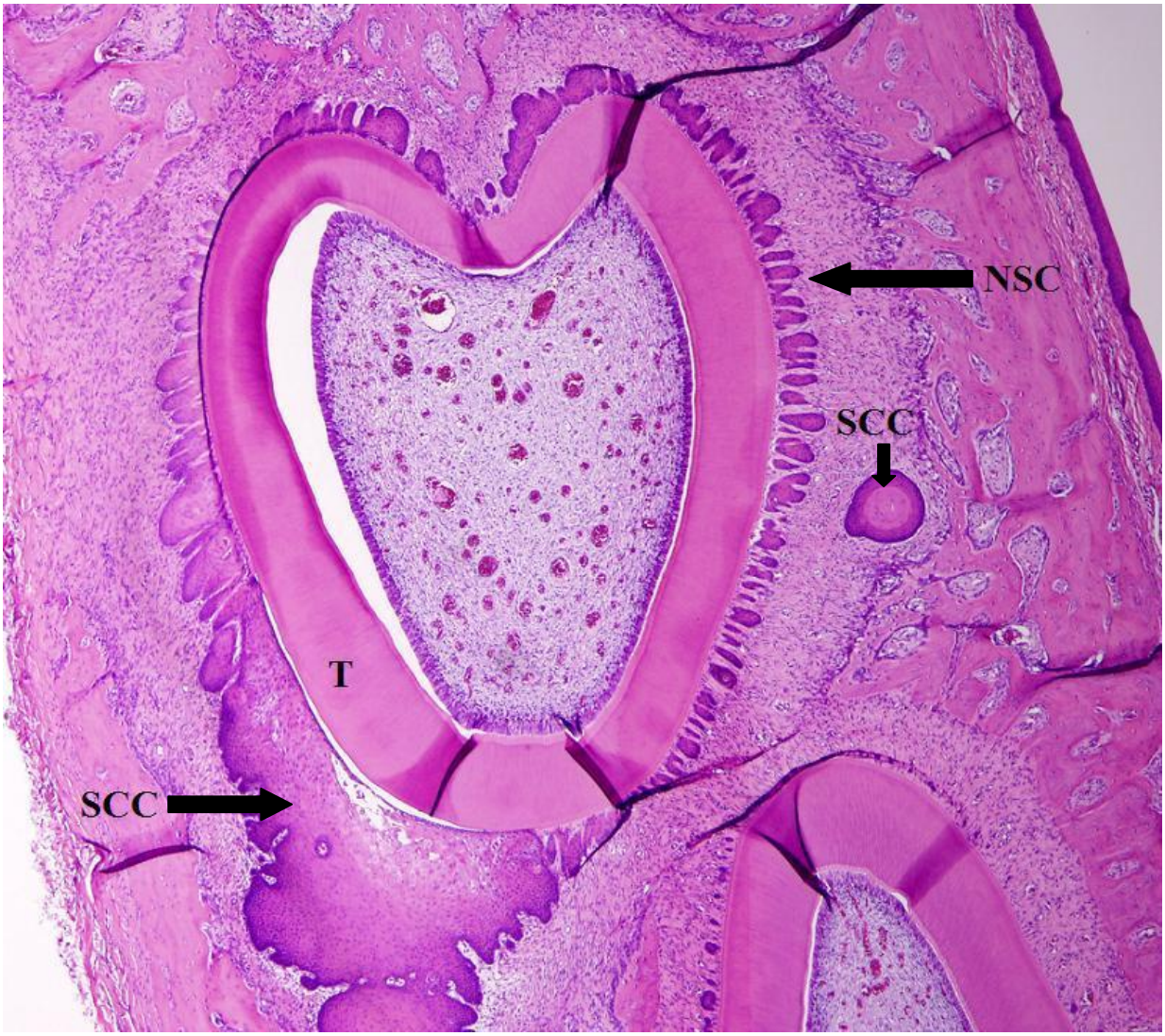


Figure 2.6 Photomicrograph of a maxilla (taken at magnification 4x) showing premolars of a female mink after 10 days of exposure. This is an example of a moderately affected jaw (lesion score 2). Surrounding the premolar teeth (T), there are two nests of squamous cell cyst (SCC, encircled top middle and bottom right) proliferation in the bone tissue.

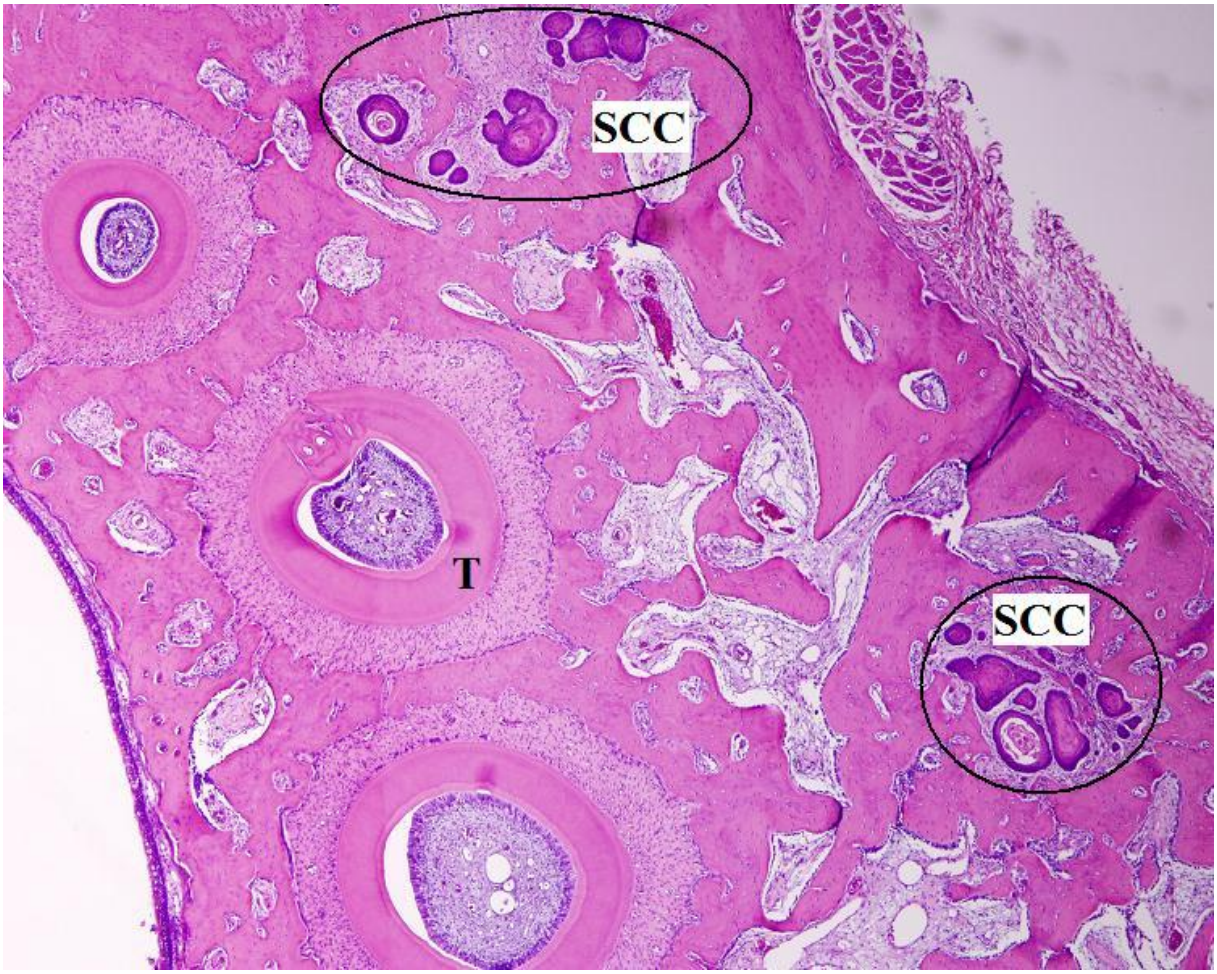


Figure 2.7 Photomicrograph of a left mandible showing molars (taken at magnification 4x) of a female mink after 14 days of exposure. This is an example of a severely affected jaw (lesion score 3). Notice the continuing proliferation of nest of squamous cells (NSC) surrounding the tooth, as well as an increased number of squamous cell cyst (SCC) proliferations throughout the bone tissue compared to Figure 2.6.

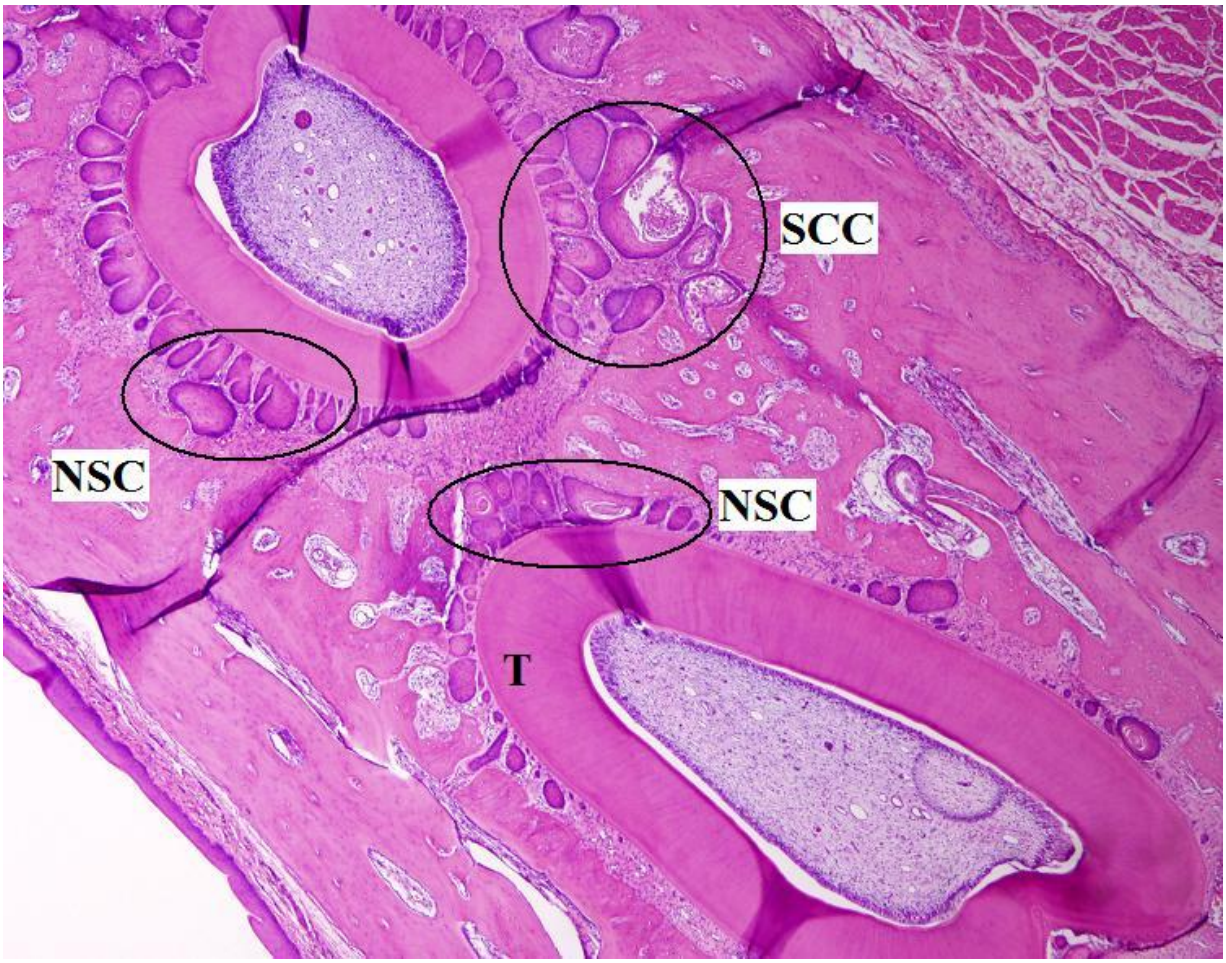


Figure 2.8 Photomicrogaph of a maxilla showing incisors (taken at magnification 4x) of a male mink after 17 days of exposure. This is an example of a severely affected jaw (lesion score 3). There are a large number of squamous cell cysts (SCC, encircled) surrounding the incisors (I) and canine (C) tooth.

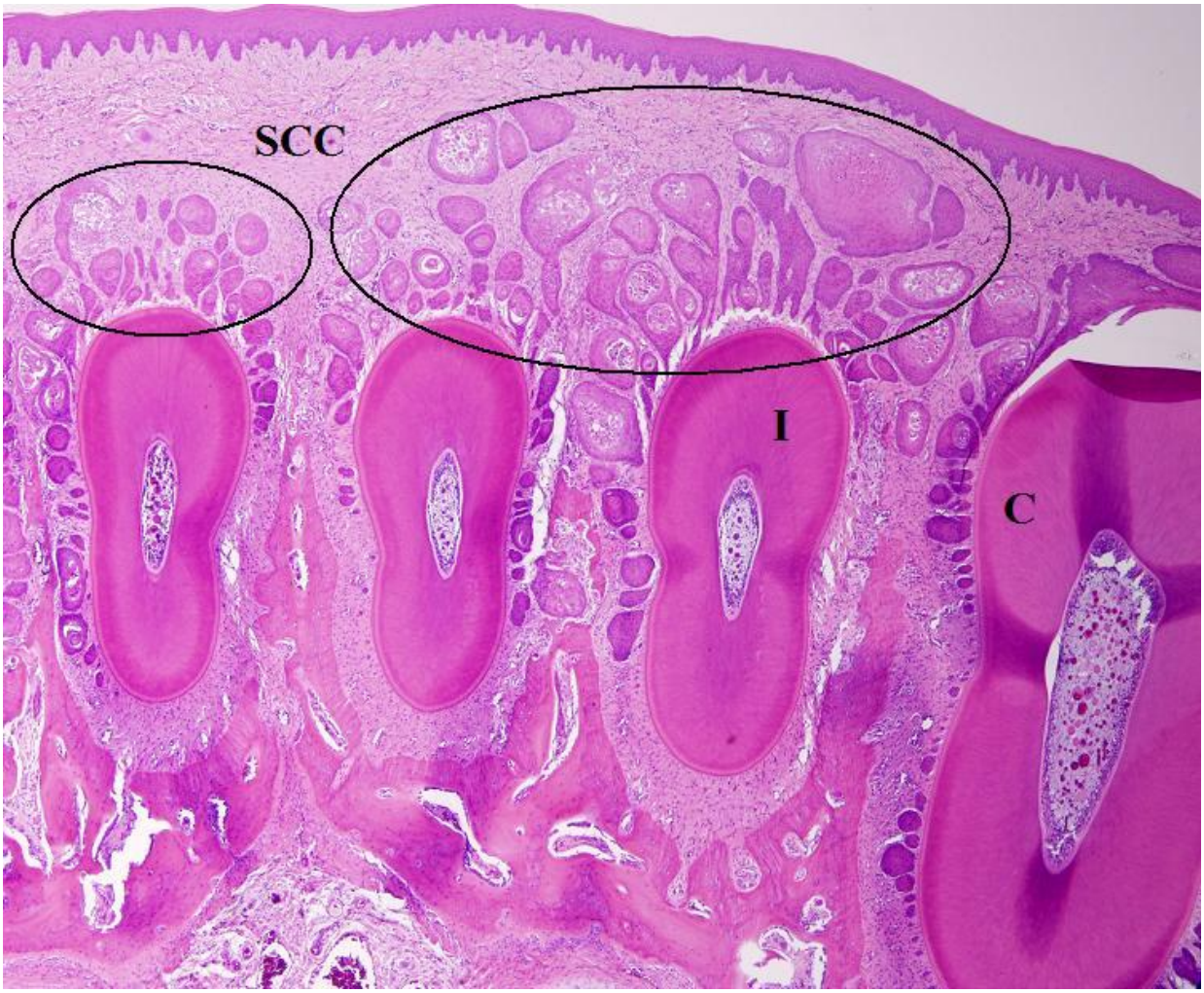


Figure 2.9 Photomicrograph of right mandible showing premolars (PM) (taken at magnification 4x) of a female mink after 21 days of exposure. This is an example of a severely affected jaw (lesion score 3). Note the loss of alveolar bone due to its replacement by squamous cell cysts (SCC), which cover the entire region of bone. Also, note that compared to Figures 2.7 and 2.8, which also show lesions with a score of 3, there are many more squamous cell cysts.

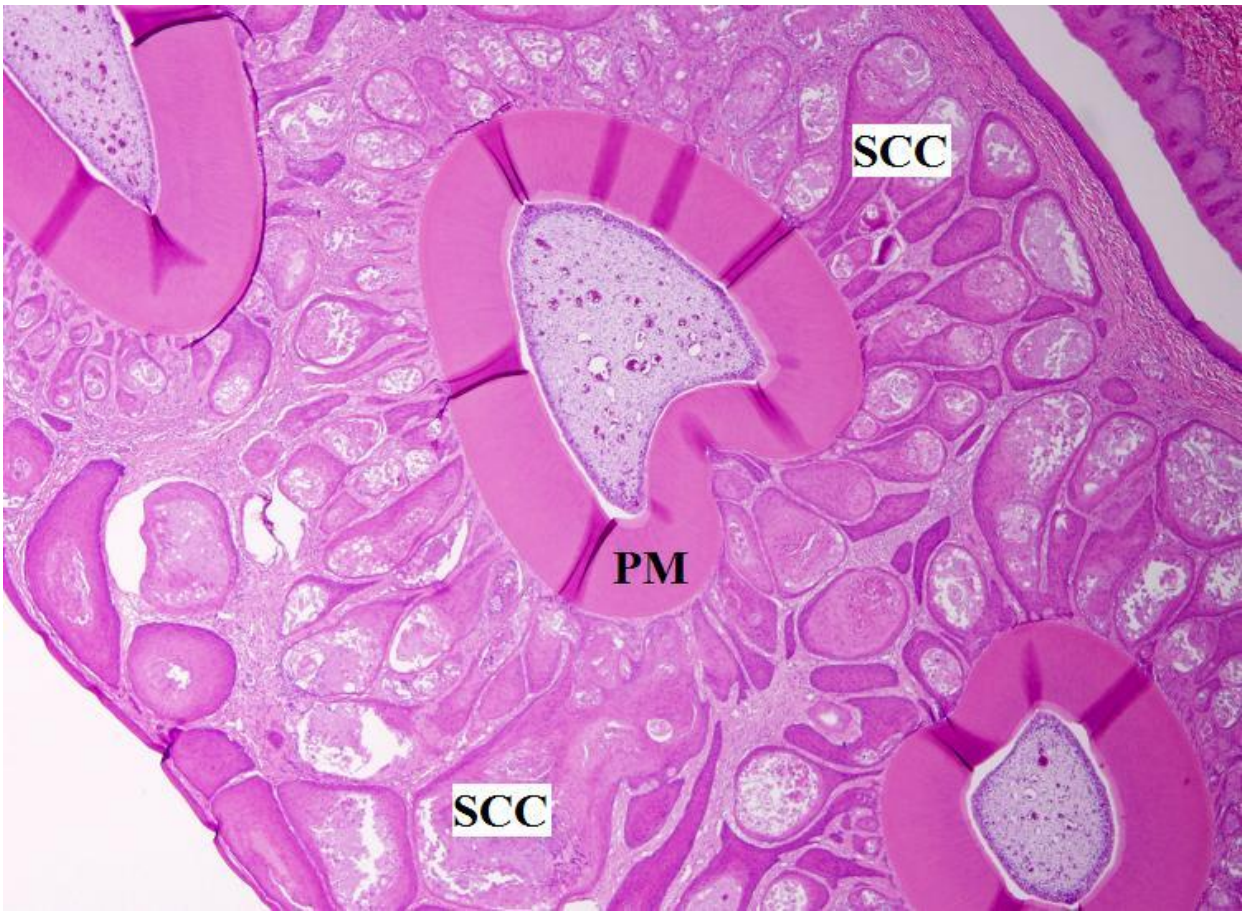
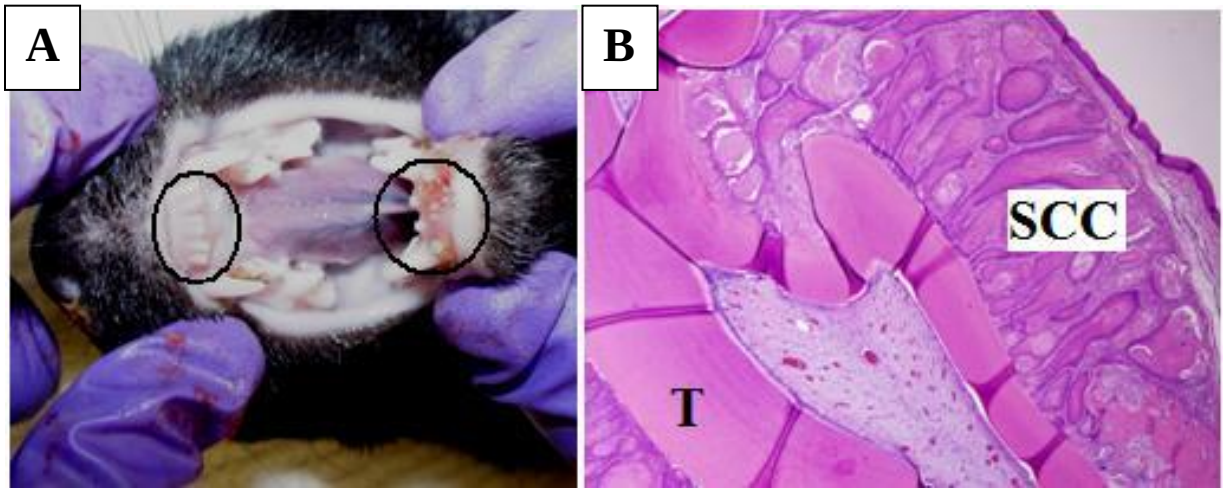


Figure 2.10 Gross (A) and histological (B) signs of the jaw lesion in a mink after 24 days of exposure to PCB 126. (A) shows crooked incisors both on the mandible and maxilla. The incisors are normally in a straight line with no gaps between the individual teeth (encircled). (B) shows the right mandibular molar (T) area (taken at magnification 4x) surrounded entirely by squamous cell cysts (SCC) (lesion score 3).



## DISCUSSION

Among the organs, only the adrenal glands showed a change in relative mass in mink fed diets containing 30 µg PCB 126/kg feed for 28 days. This increase in adrenal gland mass was also described by Hochstein et al. (1998) and Bursian et al. (2006b) in mink that were fed TCDD and other TCDD-like compounds. Increased adrenal gland mass is an indication of adrenocortical hypertrophy, which is related to overstimulation of the gland by adrenocorticotrophic hormone (ACTH) (Harvey and Sutcliffe 2010). Increase in adrenal gland mass can be due to general stress on an animal, but it is also strong evidence of stress related to toxicity. The adrenal gland is especially susceptible to toxicants due to its high fat content, extent of blood perfusion, and its potential to bioactivate toxicants catalyzed by cytochrome P450 (Verma and Rana 2009). Since both stress and toxicity can result in adrenal hypertrophy, it is difficult to determine the mechanism of action for this particular effect without histological analysis.

The development of the jaw lesion progressed very quickly during the course of the study. By 14 days of exposure, almost all mink had lesion scores of 3. However, it should be noted that the lesion continued to increase in severity beyond day 14 when the lesion score of 3 was assigned. Gross signs of the lesion, such as loose teeth, were not apparent until 21 to 28 days of exposure, which indicates there still is further development of the lesion that the current lesion classification system does not capture. This is apparent in Figures 2.5 and 2.6 where in the latter, the number of cysts is greater compared to the former even though both animals received a score of 3. Figure 2.8 shows a mandible from an animal that was exposed to PCB 126 for 21 days that was assigned a lesion score of 3.

Unexpectedly, there were two unrelated mink on day 0 that had jaws that were mildly affected (lesion score of 1) even they had not yet been exposed to PCB 126 (Table 2.2). This may indicate a level of background incidence of the lesion in the population. It was also surprising to note that one male mink necropsied after 28 days of exposure had no histological signs of the lesion. This suggests that there is genetic variation in the population of mink resulting in a few individuals being more resistant to the effects of PCB 126 compared to the rest of the population.

The present study provides an accurate description of how mandibular and maxillary squamous cell proliferation develops histologically, and also how it spreads throughout the alveolar bone tissue resulting in severe osteoporosis. While the dose used was not ecologically relevant, at 3.0 µg TEQ compared to a dose of 47 ng TEQ which was shown to induce the lesion (Bursian et al. 2005), it is assumed that the physical progression of the lesion would be similar to that described here but at a slower rate in animals exposed to lower concentrations of PCB 126.

It is recommended to expand the scoring system in order to better describe the progression of lesion severity to time of appearance of gross clinical signs. While lesion scores of 1 and 2 are well defined, a score of 3 covers a wide range of severity. A second recommendation is to develop a scoring system for the gross clinical signs such as swollen gums, bleeding gums, spreading of teeth, and loosening of teeth.

One weakness of this study is that there is not a good link between the severity of the lesion histologically and the gross signs such as swollen gums and the spreading and loss of teeth that can be observed in a live animal. Figure 2.9 shows how advanced the lesion must be histologically before severe gross signs are apparent, however photographs were only taken of severe morphological signs.

# CHAPTER 3

# THE USE OF RESVERATROL AS AN ARYL HYDROCARBON RECEPTOR ANTAGONIST TO VERIFY THE ASSUMED MECHANISM OF ACTION FOR DEVELOPMENT OF MANDIBULAR AND MAXILLARY SQUAMOUS EPITHELIAL CELL PROLIFERATION

## INTRODUCTION

One recently described effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-like compounds is a jaw lesion characterized as mandibular and maxillary squamous cell proliferation that was first described in mink (*Mustela vison*) by Render et al. (2000a,b, 2001) as a proliferation of squamous cells arising out of the gingival tissue adjacent to the teeth. These proliferations coalesce to form squamous cell cysts that invade the alveolar bone to cause osteoporosis, eventually causing loose, displaced teeth.

The presence of this lesion has been seen in wild mink trapped along the Kalamazoo River in Michigan (Beckett et al. 2005). It has also been induced in mink fed diets containing fish caught from the Saginaw River in Michigan and Housatonic River in Massachusetts in two separate studies (Bursian et al. 2006a; Bursian et al. 2006b), indicating that concentrations of TCDD-like compounds in the environment are sufficient to induce the lesion in mink. Therefore the jaw lesion is a potential biomarker for exposure to TCDD-like chemicals.

Due to the fact that the jaw lesion has been induced by TCDD-like compounds, it has thus far been assumed to have the same mechanism of action as other effects associated with TCDD-like compounds, which are mediated through the aryl hydrocarbon receptor (AhR) (Mandal 2005). This theory has not yet been tested. Other effects mediated by the AhR, including reproductive, toxicity neurotoxicity, immunotoxicity, tumor promotion, and genetic

polymorphism (Mandal 2005), have been confirmed through tests both with AhR-null mice and with the use of AhR antagonizing chemicals (Okey et al. 1994; Okey 2007).

An AhR antagonizing chemical was determined to be the best option to determine if mandibular and maxillary squamous epithelial proliferation induced in mink by TCDD-like chemicals is AhR mediated. Resveratrol (3,5,4'-trihydroxystilbene) is a known AhR antagonist, and has been used in many *in vitro* studies to block the effects of TCDD (Casper et al. 1999; Singh et al. 2000). There are fewer *in vivo* studies with resveratrol, however one study showed it to lessen the incidence of cleft palate, a teratogenic effect, in mice (Jang et al. 2008).

The hypothesis of the present study is that mink exposed to both resveratrol and 3,3'4.4'.5-pentachlorobiphenyl (PCB 126), which is the most potent TCDD-like polychlorinated biphenyl, will have less severe histological lesions than the group exposed to PCB 126 only. Assessment of hepatic ethoxyresorufin-*O*-deethylase (EROD) activity is used as an indicator of the effectiveness of reveratrol administration. EROD is a member of the CYP450 enzymes which are induced by TCDD-like compounds binding to the AhR. Activity of EROD will be decreased with the effective use of an AhR antagonist, such as resveratrol.

## METHODS

Male and female mink, five months of age, were assigned to one of four treatment groups: vehicle control, PCB 126, resveratrol, or resveratrol plus PCB 126. Since resveratrol has not previously been used in mink, a group of animals exposed only to resveratrol, as well as a group of animals dosed with the vehicles to show that there were no compounding effects of the administration procedures (gavage and IP injection) were included.

The resveratrol (Sigma-Aldrich, St. Louis, MO) was suspended in 4% carboxymethycellulose (Sigma-Aldrich, St. Louis, MO) at a concentration of 20 mg resveratrol/ml. PCB 126 (certified 99.7% pure, AccuStandard. New Haven, CT) was dissolved in hexane and then diluted with corn oil at a concentration of 50 µg PCB 126/ml corn oil. Reseveratrol was administered via gavage for six consecutive days at a rate of 2.5 ml resveratrol suspension/kg bw/day resulting in a dose of 50 mg resveratrol/kg bw/day. Control mink were gavaged with 2.5 ml 4% carboxymethylcellulose/kg bw/day for six consecutive days. PCB 126 (30 µg /kg bw) was administered to the mink by intraperitoneal (IP) injection (0.6 ml/kg bw) on the fifth day of resveratrol treatment, approximately one hour after administration of resveratrol. Mink not receiving PCB 126 were given an IP injection of corn oil. The dosing regime was based on Jang et al. (2008). The dose of PCB 126 was chosen because it had previously been shown to induce the lesion in mink with this route of exposure. I pilot study was conducted in which six adult female mink were used to determine whether IP injection and gavage were efficatious routes of exposure in terms of inducing the lesion. Four mink were administered an IP injection of PCB 126 at a dose of 30µg/kg bw, and two of them were administered a second dose one week later. Two mink were given a dose of PCB 126 (30 µg/kg bw) via gavage and one of them was given a second dose one week later. Nine weeks later the animals were necropsied and all andimals showed histological signs of the lesion.

Mink were housed singly in wire cages (61 cm length, 25.5 cm width, 38 cm height) with attached wooden nest boxes in an open-sided pole barn. Animals were provided a standard ranch diet (Table 2.1) and water ad libitum.

Nine weeks after PCB 126 administration, the mink were euthanized with $CO_2$, and the thyroid and adrenal glands, kidneys, spleen, heart, liver, and brain were removed, weighed, and

placed in 10% neutral buffered formalin. Portions of the livers were removed and immediately frozen in liquid nitrogen and were placed in a -80°C freezer for subsequent determination of EROD activity. The jaws were removed, stripped of adhering skin and tissue and placed in 10% neutral buffered formalin (VWR International, West Chester, PA) for subsequent processing. Jaws then were placed in Rapid Decal (Leica Biosystems Peterborough, Peterborough, UK) for 36-48 hours for decalcification. After decalcification, jaws were trimmed, embedded in paraffin, sectioned (thickness, 5µm) and stained with hematoxylin and eosin. The slides were examined under a light microscope by a board certified pathologist for signs of the jaw lesion. The severity of the lesion was described on a scale of 0 to 3, as described in Beckett et al. (2005).

**EROD assay**

Assessment of hepatic EROD activity was according to Kennedy and Jones (1994). Portions (approximately 0.5 g) of the liver were weighed and placed in Tris buffer (0.05 M tris(hydroxymethyl)aminomethane [Sigma Aldrich, St. Louis, MO], 1.15% KCl [Sigma Aldrich] in nanopure water, pH 7.5 at 4°C). Livers were then homogenized and centrifuged for 10 minutes at 10,000 x g in a Sorvall RC 6 Plus centrifuge (Thermo Fisher Scientific, Asheville, NC) with an SM24 rotor. The supernatant was collected and placed in an ultracentrifuge tube (Polyallomer Thick Wall, 16 x 17 mm, 13.5 ml capacity, Beckman Coulter, Brea, CA) with enough Tris buffer to bring the total mass of the tube and the contents to 12.3 g. This was then spun for 30 minutes in a Beckman L8-80 M centrifuge (Beckman Coulter) at 100,000 x g (33,300 rpm in a 50TI rotor) at 4°C. The supernatant was discarded, and the pellet was re-suspended in EDTA buffer (10 mM EDTA; JT Baker, Phillipsburg, NJ), 1.15 % KCl in nanopure water, pH 7.4) and then transferred to a new ultracentrifuge tube. EDTA buffer was added to bring the total mass to 12.3 g. This was spun again at 100,000 x G for 60 minutes at 4 °C. The

supernatant was discarded, and the pellet was re-suspended in 0.5 mL Microsome Stabilizing Buffer (20% glycerol, 0.1 M $KH_2PO_4$, [J.T. Baker] 1.0 mM EDTA, 1.0 mM dithiothreitol [DTT, Sigma Aldrich], pH 7.25). This mixture was then homogenized, placed into Eppendorf tubes (Sarstedt, Nuembrecht, Germany), and stored in a -80 freezer until analysis for EROD activity.

EROD activity was measured in the following assay, which uses ethoxyresorufin as a substrate. The reagents were added to a 96-well plate in the following order: HEPES buffer (0.05M, pH 7.8 at 37°C; 108 µl for blanks and 78µl for samples); 3 µl of thawed microsomes; substrate (30 µL of a 5 µM solution of resorufin ethyl ether [Sigma Aldrich] in methanol. The plate was then incubated for 10 minutes at 37 °C. To begin the reaction, 30 µL of a 2 mM NADPH (Sigma Aldrich) solution in HEPES buffer was added to each of the sample wells. The plate was placed into the fluoroscan (Fluoroscan Ascent, Thermo Electron Corporation, Vantaa, Finland) and fluorescence readings were taken every 2 minutes for 30 minutes to measure the rate of reaction (538/590 nm filter). The plate was then removed from the fluoroscan, and 60 µL fluorescamine (Sigma Aldrich) dissolved in acetonitrile (Burdick and Jackson, Muskegon, MI) was added to each well to stop the reaction. The plate was covered and incubated for 10 minutes before a final reading to measure the amount of protein in each well (405/460nm filter).

A standard curve was determined, using bovine serum albumin (BSA) for the protein, and a solution of resorufin sodium salt in methanol for total resorufin concentration, measured by fluorescence. The concentrations of BSA in the wells were 0, 0.012, 0.024, 0.036, 0.048, 0.106 mg BSA/well. The concentrations of resorufin for the standard curve were 0, 7.5, 15, 60, 120, and 210 pmol/well. Each well was measured in triplicate.

The slope of the curve, which indicates change from ethoxyresofurin to resorufin, was measured in pmol/min. The slope is divided by the mg of protein that was measured in each well to give the final value of EROD activity in pmol/min/mg.

### Statistical Methods

To increase statistical power to detect changes in jaw lesion incidence and severety, a proportionally imbalanced 2x2 factorial design was used. Eight litters of four mink and 15 litters of two mink that contained at least one of each sex were selected for this project. The kits were randomly assigned to a treatment group so that each treatment was represented within each litter for a randomized block design. The Proc Mixed function in SAS was used. A Tukey's test was done to test for significant differences between the treatments due to the unbalanced design. Fixed effects for the model were treatment, and sex, and random effects for the model were litter and bodyweight.

## RESULTS

### Organ Mass

There were no significant differences in bodyweight brain, heart, kidney, adrenal gland, or thyroid gland masses among the treatment groups (Table 3.2). Only liver and spleen masses were significantly different among the groups (Table 3.2, Figures 3.1 and 3.2). For the liver, treatment ($p < 0.0001$) and sex ($p = 0.0085$) were significant fixed effects (females had decreased liver mass compared to males). Liver masses of the the mink in the PCB 126 and PCB 126 plus resveratrol groups were significantly greater ($p < 0.0001$) compared to the control and resveratrol groups. There were no differences between the PCB 126 and PCB 126 plus resveratrol groups nor were there differences between the control and reseveratrol groups. For the spleen, there

were significant fixed effects of treatment ($p = 0.0004$). As with the liver, spleen masses of the mink in the PCB 126 and PCB 126 plus resveratrol groups were significantly greater ($p < 0.0031$) compared to the control and resveratrol groups. There were no differences between the PCB 126 and PCB 126 plus resveratrol groups nor were there differences between the control and reseveratrol groups. Litter was not a significant effect for any organ weights.

Table 3.1 Mean organ mass (g) of mink dosed with PCB 126, resveratrol or a combination of PCB 126 and resveratrol

| Organ | Control (n = 8) | Resveratrol (n = 8) | PCB 126 (n = 23) | PCB 126 + resveratrol (n = 23) |
|---|---|---|---|---|
| Thyroid gland | 0.094 ± 0.016[1] | 0.042 ± 0.016 | 0.059 ± 0.0091 | 0.059 ± 0.0092 |
| Adrenal glands | 0.10 ± 0.0094 | 0.093 ± 0.0097 | 0.120 ± 0.0055 | 0.120 ± 0.0056 |
| Kidneys | 8.68 ± 0.82 | 7.73 ± 0.85 | 9.44 ± 0.48 | 8.32 ± 0.49 |
| Heart | 8.24 ± 0.27 | 7.69 ± 0.28 | 8.08 ± 0.27 | 7.74 ± 0.16 |
| Spleen | $2.53^{a}$ ± 0.42 | $2.32^{a}$ ± 0.43 | $4.22^{b}$ ± 0.26 | $3.81^{b}$ ± 0.26 |
| Brain | 9.05 ± 0.21 | 8.93 ± 0.21 | 9.02 ± 0.12 | 9.09 ± 0.12 |
| Liver | $54.8^{a}$ ± 3.7 | $49.2^{a}$ ± 3.8 | $78.5^{b}$ ± 2.1 | $75.7^{b}$ ± 2.1 |
| Bodyweight | 3273±165 | 3585±165 | 3267±96 | 3148±96 |

[1]Data presented as mean± SE. Means with different superscripts are significantly different from on another at $p < 0.05$

**EROD activity**

EROD activity was significantly higher in both treatment groups that were exposed to PCB 126, compared to the control ($p < 0.0001$). There was no significant difference between the control and resveratrol groups nor between the PCB 126 and PCB 126 plus resveratrol groups (Table 3.2). Both sex and litter were significant fixed effects, but no interactions were significant, and there was ultimately no effect of these differences on the treatment effects. Sex was shown to be a significant effect and was included as a covariate in the model, along with litter. While there were significant differences between sexes for EROD activity (female activities were higher than male activities) within the treatment groups, this did not have an overall effect on the differences in EROD activities between the treatment groups (Figure 3.1).

**Pathology**

Nine weeks after administration of six daily doses of 50 mg resveratrol/kg body weight and/or a single intraperitoneal dose of 30 µg PCB 126/kg body weight, jaw tissues from control animals and mink administered resveratrol only were normal. One animal in the PCB 126 group had a lesion score of 2 while the other 22 animals had lesion scores of 3. Two mink in the PCB 126 plus resveratrol group had lesion scores of 2 while the other 21 animals had lesion scores of 3 (Table 3.3). Figure 3.2 presents photomicrographs of the mandibular premolar region of four littermates in the control, reveratrol, PCB 126 and PCB 126 plus resveratrol groups.

Figure 3.1 Mean hepatic EROD activity (pmol/min/mg) ± SE in mink dosed with PCB 126, resveratrol, or a combination of both. All values are statistically significant ($p < 0.005$), different superscripts are significantly different from one another at $p < 0.05$.

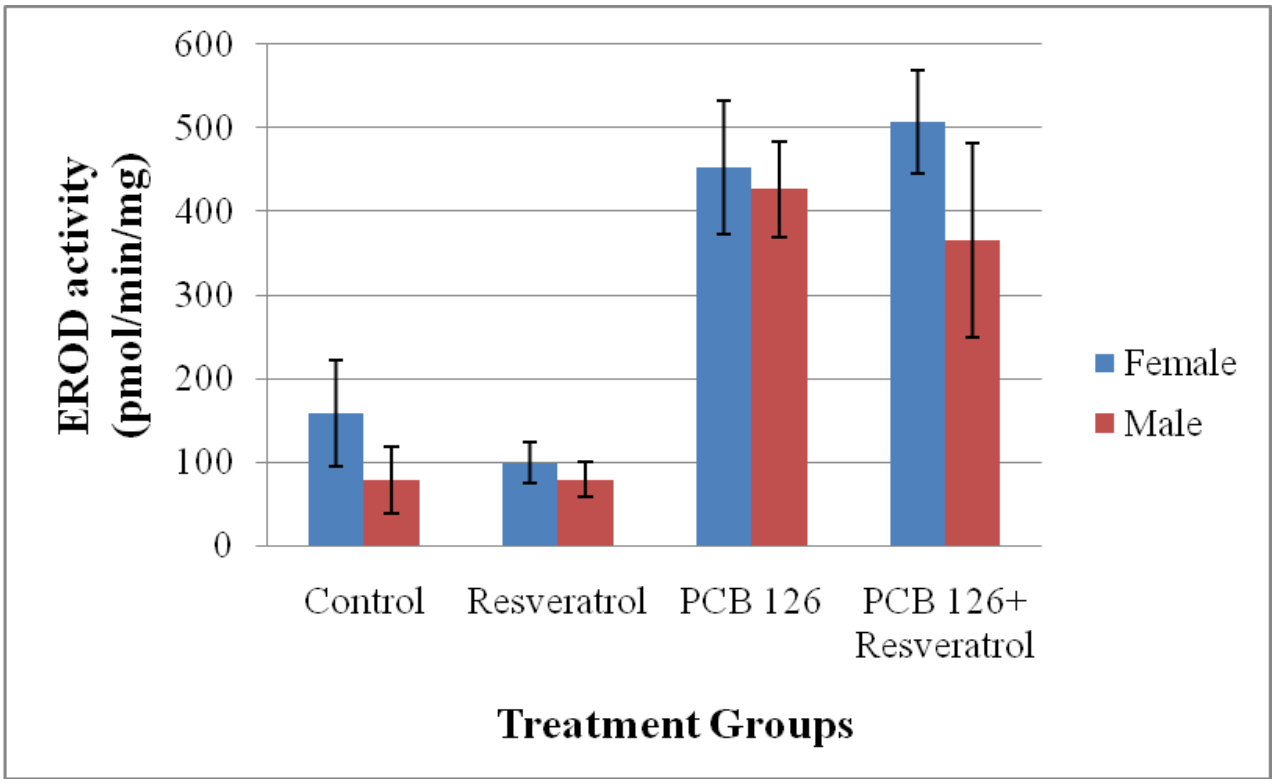


Table 3.2 Mean hepatic EROD activity ± SE in mink dosed with PCB 126, resveratrol or a combination of PCB 126 and resveratrol.

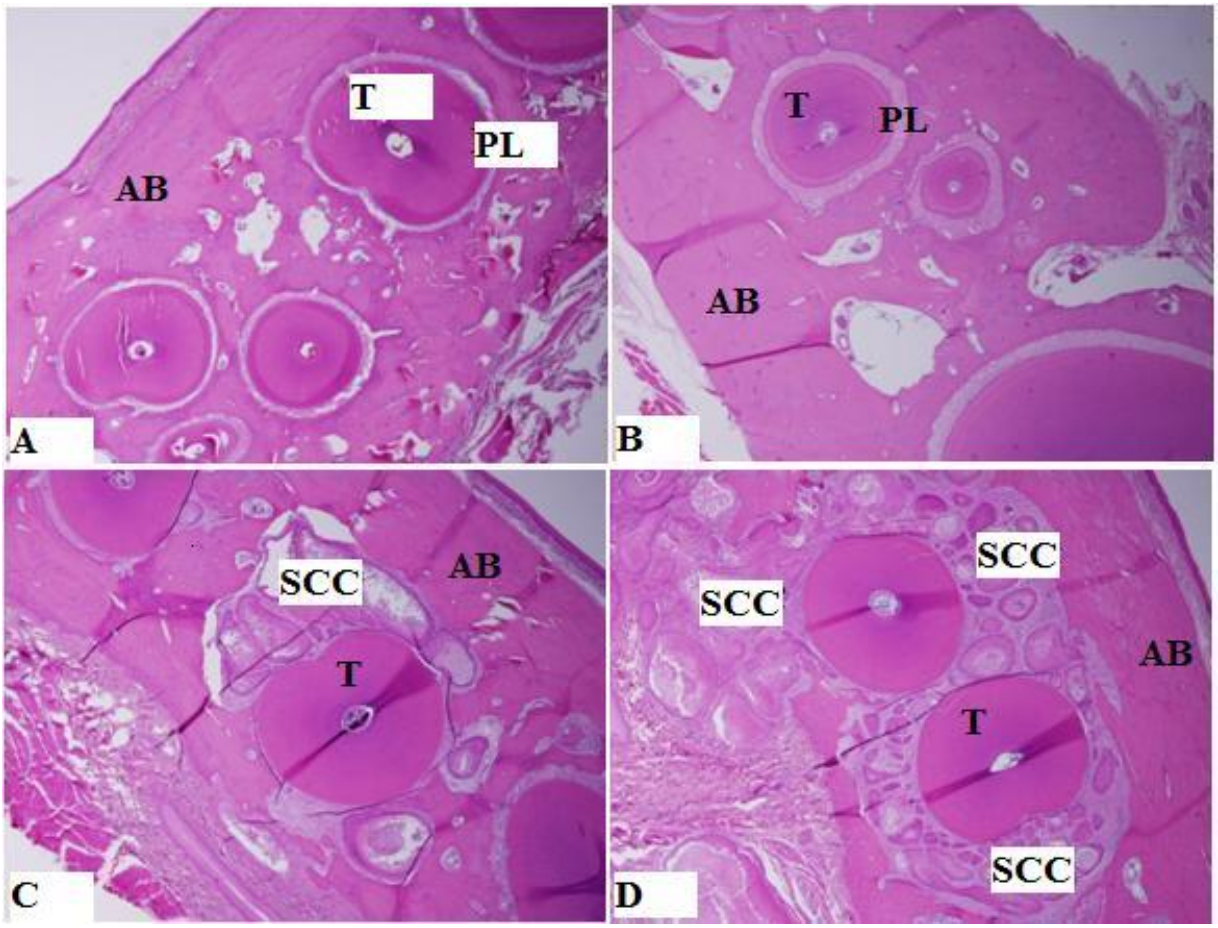
| EROD activity (pmol/min/mg) | | | |
|---|---|---|---|
| Control (n = 8) | Resveratrol (n = 8) | PCB 126 (n = 22) | PCB 126+ Resveratrol (n = 23) |
| $117\pm28^{a_1}$ | $83\pm28^{a}$ | $438\pm25^{b}$ | $435\pm24^{b}$ |

[1]All values are statistically significant, ($p < 0.005$), different superscripts are significantly different from on another at $p < 0.05$

Table 3.3 Summary of jaw lesion scores in mink dosed with PCB 126, resveratrol or a combination of PCB 126 and resveratrol

| Lesion Score | Control (n = 8) | Resveratrol (n = 8) | PCB 126 (n = 23) | PCB 126+ resveratrol (n = 23) |
|---|---|---|---|---|
| 0 | 8 | 8 | 0 | 0 |
| 1 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 1 | 2 |
| 3 | 0 | 0 | 22 | 21 |

Figure 3.2 The photomicrographs show mandibular squamous epithelial proliferation in premolar regions of mink nine weeks after administration of six daily doses of 50 mg resveratrol/kg body weight and/or a single intraperitoneal dose of 30 µg PCB 126/kg body weight. (A) Photomicrograph of a control animal. Note the preemolar teeth (T), peridontal ligament (PL), and aveolar bone (AB). (B) Photomigraph of an animal dosed with resveratrol, with no abnormalities or evidence of squamous cell cysts. (C) Photomigrograph of an animal dosed with PCB 126, which was assigned a lesion score of 2, due to the squamous cell cysts (SCC) surrounding the tooth. (D) Photomicrograph of an animal dosed with PCB 126 plus reseveratrol, which was assigned a lesion score of 3. Compared to (C) there is a greater number of squamous cell cysts (SCC) in the tissue surrounding the tooth.



## DISCUSSION

Increase in liver mass in mink, is a common effect observed in other species resulting from exposure to TCDD-like compounds. Therefore it is expected that the livers of those mink exposed to PCB 126 to be enlarged (Restum et al. 1998; Beckett 2005). The fact that there was no significant difference in liver mass between the group treated with PCB 126 plus resveratrol and the PCB 126 only group indicates that the resveratrol was not effective in ameliorating the general toxic effects of PCB 126 in mink. Spleen mass has also been shown to be increased when mink are exposed to PCBs (Restum et al. 1998), which is another indication of toxicity. However, in a study in which mice were exposed to both TCDD and resveratrol, the TCDD-treated mice had a decrease in spleen mass as a percent of body weight but not in the TCDD plus resveratrol treatment group (Ishida et al. 2009). The authors offered no explanation for this apparent disparity.

There were no significant differences in EROD activity between the two groups of mink that were exposed to PCB 126, which had significantly greater activity compared to the two groups of mink that were not dosed with PCB 126. This indicates that PCB 126 induced CYP1A1 and that resveratrol did not block the induction. There was wide variation in EROD activity, not only within treatment groups, but also within the triplicate of the same animal. even though the total amounts of protein and resorufin measured were very similar. However, there was a greater numerical difference between the averages for the control and resveratrol treatments (99.94 and 67.64, respectively) compared to the difference in averages between the PCB 126 and PCB 126 plus resveratrol treatments (440.15 and 434.50, respectively). A study with human liver cells showed a similar result, in which resveratrol inhibited EROD slightly, but

not significantly (Chun et al. 1999). The variability of the data could partially explain the reason why there were no statistically significant differences between the groups.

The measurement of EROD activity is a well-established approach to assess the induction of the cytochrome P450 1A1 (CYP1A1) liver enzymes (Moore et al. 2009). I*n vitro* studies have shown that resveratrol competitively inhibits TCDD-induced EROD activity in a dose dependent manner, and that if the dose of resveratrol is great enough, the effect of TCDD on EROD activity can be completely blocked (Ciolino et al. 1998; Ciolino and Yeh 1999). *In vivo* studies, however, failed to show the same attenuating effects on EROD activities measured in mice treated with both resveratrol (administered orally at a dose of 20 mg/kg bw/day for 28 days) and PCB 126. Mice treated with the combination of resveratrol and PCB 126 did not have lower EROD activities compared to mice treated with PCB 126 only. However, when resveratrol was administered via subcutaneous injection at a dose of 225 mg/kg bw/day for 5 days, the effect of TCDD on EROD activity was decreased by approximately 23% (Ishida et al. 2009). The latter response could be due to the increase in the amount of resveratrol administrered, the route of administration, or exposure duration. This may indicate than in an *in vivo* model, EROD activity is an effective tool to determine whether resveratrol is counteracting the effects of TCDD-like compounds only once effective doses of both the agonist and antagonist have been established.

As shown in Table 3.3, there was no difference in lesion severity between the PCB 126 and PCB 126 plus resveratrol treatment groups. However, the lack of an effect of resveratrol on the occurrence and severity of the PCB 126-induced jaw lesion is supported by both the organ mass and EROD activity data. While there appears to be a range in the severity of those lesions assigned a score of 3 (as shown in Figure 3.1), they are not consistent with differences in treatment. When apparent differences in lesion severity were found, it was not always the animal

treated with resveratrol that had the less severe lesion. There appears to be no relationship between resveratrol treatment and the severity of lesions given a score of 3.
Resveratrol has been used effectively *in vivo* to study a similar effect, the incidence of TCDD-induced cleft palate in mice (Jang et al. 2008). In this study, the incidence of cleft palate in fetuses exposed to resveratrol *in utero* for six consecutive days followed by a single dose of TCDD was reduced by 18.4% compared to fetuses exposed to TCDD only.

There are several possible reasons why resveratrol was not effective in preventing or diminishing the inducement of mandibular and maxillary squamous epithelial cell proliferation by PCB 126 in the present study. Suspension of resveratrol in carboxymethylcellulose and administration via gavage was judged to be the most effective route of administration based on the limited solubility of resveratrol; however it is possible that resveratrol was metabolized before it reached the site of action. In contrast, PCB 126 was easily injected into the animals, which is a more efficacious route of exposure because it does not go through first-pass metabolism in the liver. However, the dose of PCB 126 used may have been so high that it out-competed the resveratrol. This was first discovered in a preliminary experiment, in which five female mink were injected with PCB 126 and then necropsied nine weeks later. It was shown that all of these mink exhibited histological signs of the lesion. This is the first time in which the lesion has been induced by this method, and shows that contact of PCB 126 with the gingival tissue is not necessary for inducement of the jaw lesion. Furthermore, PCB 126 is much more persistent within the animal, and as a result may have been able to activate the AhR once the resveratrol had been metabolized. These assumptions require further research to determine if there are other more effective routes of exposure for resveratrol. Necropsy of the animals nine weeks after treatment may have been too long, allowing the lesion to develop after the

resveratrol had been metabolized. It is possible that examination of the animals shortly after initiation of the lesion would have indicated a protective effect by resveratrol. Thus, the results of the organ mass analysis, the EROD assay, and the histological analysis of the jaws all indicate that short-term exposure to resveratrol was not effective in preventing the inducement of the jaw lesion by PCB 126 in mink.

CHAPTER 4

# COMPARISON BETWEEN PCB 126-INDUCED MANDIBULAR AND MAXILLARY SQUAMOUS EPITHELIAL PROLIFERATION AND A LESION OF BACTERIAL ORIGIN IN BLUE IRIS MINK

## INTRODUCTION

Many species of wildlife are reported to have gross clinical signs indicative of a jaw lesion similar to those reported for mink (*Mustela vison*) exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-like chemicals. Mortenson et al. (1992) described "skull-bone lesions" in harbor seals in the Baltic region, which included "loss of alveolar bone tissue around individual teeth" or "considerable loss of alveolar bone around teeth and other parts of the jaw, as well as multiple loss of teeth." Beland et al. (1993) and De Guise et al. (1995) both reported tooth loss and periodontitis in beluga whales found in the St. Lawrence Estuary, and Sonne et al. (2007) reported similart effects in polar bears in Greenland. A clear cause of these jaw bone effects has not been established, but polychlorinated biphenyls (PCBs) and other polychlorinated hydrocarbons (PCHs) are known to affect high trophic-level preditors such as seals, whales and polar bears. The presence of these chemicals has been documented in the environments mentioned above and it is possible that these chemicals are responsible for the reported lesions.Therefore it is possible that a lesion induced by 3,3',4,4',5-pentachlorobiphenyl (PCB 126), such as has been described in mink (Render et al. 2000a, b, 2001; Beckett et al. 2005; Bursian et al. 2006a, b; Haynes et al. 2009), could be similar to the condition described in other wildlife species.

In a recent report Hammer et al. (2005) describes an oral lesion in a color strain of ranch mink that appears to be very similar to the condition reported in mink exposed to TCDD-like chemicals. Blue iris mink can develop severe destructive periodontal disease, which results in the loss of teeth. These mink are susceptible to an autosomal disorder known as Chediak-Higashi syndrome (CHS), which is associated with a deficiency of the immune system. This makes them more susceptible to various infections including skin abscesses (Hammer et al. 2005). The authors of this report studied the association between destructive periodontal disease (measured by radiographic images of mink jaws) and counts of abnormal polymorphonuclear leukocytes (which indicates Chediak-Higashi syndrome). It was concluded that tooth loss was related to destructive periodontal disease, although a significant relationship to the number of CHS leukocytes was not shown.

The purpose of the present study was to compare histologically jaws from mink exposed to PCB 126 and those from blue iris mink that had bleeding gums and looose and/or missing teeth to determine if the oral lesions were identical. If so, it is possible that TCDD-like chemicals could be inducing immunosuppression, which in turns renders exposed mink susceptible to bacterial infection, resulting in severe periodontal disease.

## METHODS

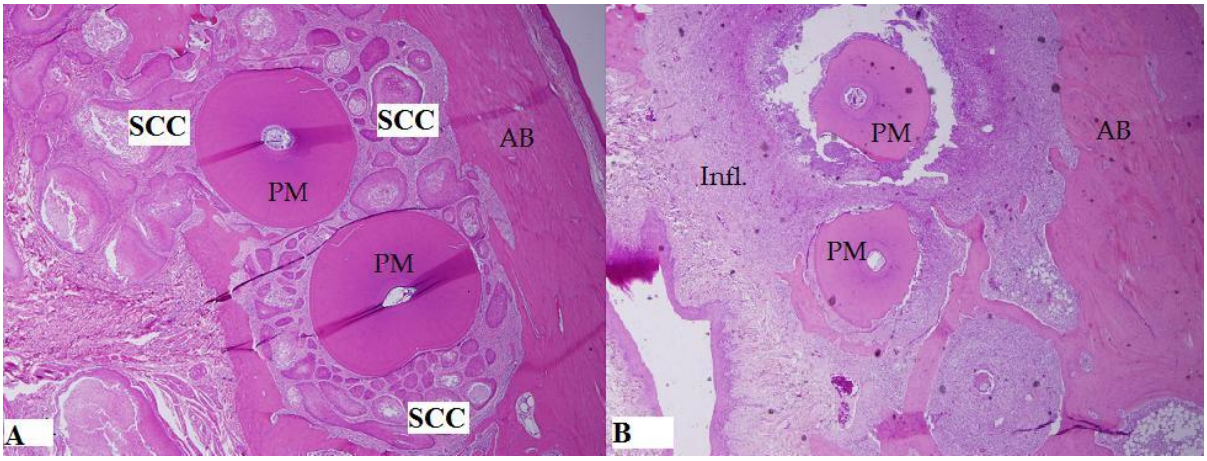
The jaws of six blue iris mink having gross clinical signs of periodontal disease were collected at the termination of an unrelated trial. Mink were euthanized with $CO_2$ and the jaws were removed, stripped of adhering skin and tissue and placed in 10% neutral buffered formalin (VWR International, West Chester, PA) for subsequent processing. Jaws then were placed in Rapid Decal (Leica Biosystems Peterborough, Peterborough, UK) for 36-48 hours for decalcification. After decalcification, jaws were trimmed, embedded in paraffin, sectioned

(thickness, 5µm) and stained with hematoxylin and eosin. The slides were examined under light microscope by a board certified pathologist for signs of the jaw lesion.

## RESULTS AND DISCUSSION

The jaw tissue from the blue iris mink had lesions indicative of lymphoplasacytic gingivitis and osteomyelitis, caused by immunosuppresion and inflammation entering the dental sulcus (Figure 4.1). Despite the similarity in gross clinical signs between the blue iris mink and mink exposed to PCB 126, histological examination of jaw tissue indicated that the inflammatory lesions in the blue iris mink were unrelated to the squamous cell cysts that are indicative of a proliferative/neoplastic condition. While the destruction of the alveolar bone appears similar in the the blue iris mink and mink dosed with PCB 126, the underlying cause is different. One additional difference is that the inflammatory destruction also affects the teeth, whereas the proliferative lesions destroy only the surrounding alveolar bone, leaving the teeth intact. Gross lesions seen in seals, whales, polar bears could be indicative of either exposure to TCDD-like compounds or an inflammatory response. The results of this study suggest that histological examination of jaw tissue collected from wildlife species will therefore be necessary to dfifferentiate between the two conditions.

Figure 4.1: Photomicrograph of the mandible showing premolars from a mink exposed to PCB 126 (A) and a mandible of a blue iris mink (B). Notice the squamous cell cysts (SCC) surrounding the tooth in (A), which destroy the surrounding alveolar bone (AB) but not the premolar teeth (PM). Compare this to (B) which shows destructive inflammation (Infl.) that destroys both the alveolar bone and premolar teeth. Both result in severe bone loss but are histologically distinct effects.



# CHAPTER 5

## RECOMMENDATIONS FOR FUTURE RESEARCH

Given the problems with our studies, I would like to offer suggestions for refining them. While the timeline study offered good descriptive information about the development of the lesion, the study was not designed in such a way as to provide information to correlate it to concentrations of PCB 126 in the tissues. I recommend repeating the study, but with minor changes. The study should be started in the fall so that age of mink does not provide such a complicating factor. Organs should be analyzed for concentrations of PCB 126, and controls should be used so that the organ mass data can be properly analyzed. Further immunohistochemistry can be done to determine whether the lesion is neoplastic by searching for a compatible

For the resveratrol study, it may be a good idea to develop the Harlan Sprague-Dawley rat as a model for studying the jaw lesion, as they have been shown to develop this squamous cell carcinoma with chronic exposure to TCDD-like compounds (Yoshizawa, 2005). Given the expense of resveratrol, it would be much more affordable and convenient to repeat the resveratrol experiments with rats compared to mink.

To follow up with the comparison between the PCB 126-induced jaw lesion and the skull-bone lesions described in other species of wildlife, it would be valuable to analyze their tissues histologically to determine if they are suffering from periodontitis or are developing squamous cell cysts.

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