DECIPHERING THE LINK BETWEEN POLYCHLORINATED BIPHENYLS, IMMUNE FUNCTION AND EXERCISE

Mahesh R. Pillai

A Dissertation

Submitted to the Graduate College of Bowling Green State University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2017

Committee:

Lee A. Meserve, Advisor

Howard C. Cromwell
Graduate Faculty Representative

Todd K. Keylock

Scott O. Rogers

Vipaporn Phuntumart
ABSTRACT

Lee A. Meserve, Advisor

Polychlorinated biphenyls (PCBs) are environmental pollutants and endocrine disruptors, harmfully affecting reproductive, endocrine, neurological and immunological systems. This has implications for processes such as wound healing, which is modulated by the immunological response of the body. Conversely, while PCBs can be linked to diminished wound healing, outside of PCB pollution systems, exercise has been shown to accelerate wound healing. However, the potential for moderate intensity exercise to modulate or offset the harmful effects of a toxin like PCB are yet unknown. Exploration of this possible moderation on local immune response was achieved by measuring wound size and analyzing the concentrations of proinflammatory cytokines, interleukin-1β (IL-1β), interleukin-6 (IL-6), keratinocyte chemoattractant (KC), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-α (TNF-α) in wounds (inflicted by punch biopsy) in mice that were not exercised as compared with those previously exercised at moderate intensity by running on a treadmill for 30min/day and then injected intraperitoneally with Aroclor 1254 (industrial mixture of PCB congeners) in doses of either 0, 100, 500 and 1000 ppm (wt/wt of mice). Mice were euthanized at Day 3 or Day 5 (n = 3-6) and skin excised from the wound area was homogenized and analyzed for cytokine content. Systemic effects of exercise on immune function in PCB exposed animals were examined by lipopolysaccharide (LPS) challenge (intraperitoneal injections) and analyzed by measuring the average body temperatures using a thermal imaging camera. Wound healing data revealed that in animals not exercised only the greatest dose of PCB (1000 µg/g) showed a pattern for faster wound healing. Exercise produced a pattern of more rapid wound healing rates compared to the animals administered similar doses, except for animals administered 100 µg/g PCB. Concentrations of pro-inflammatory cytokines revealed patterns that could explain many of the changes observed.
in the wound healing rates and could be used as a good predictor of progression of wound
healing. Average body temperature measurements revealed a febrinergic response to LPS
stimulation only in the mice not exercised that were not administered PCB. In exercised mice this
response was blunted, indicating lowered inflammatory response. Overall, exercise appears to
have a beneficial effect on wound healing and LPS stimulation, only in animals not administered
PCB. Recent studies show both PCB and exercise impair functioning of macrophages and that
could have produced the similarity of results in the present study and needs to be further explored.
Dedicated to my grandparents, Mrs. Chandrika and Mr. Sankaranarayanan Nair and parents, Mrs. Prema and Mr. Ramachandran Pillai

for their constant love and encouragement and

my loving brother, Late Dr. Nilesh Pillai, may his soul rest in peace.
ACKNOWLEDGMENTS

I am extremely grateful to my PhD advisor Dr. Lee A. Meserve, who has been the kindest and most understanding person I could have asked for. Through all my personal and research problems, he has constantly stayed positive and provided all the support without any reservations. Him and his wife, Marge have always treated us as family and always made us feel welcome at their home. Dr. Meserve is a wonderful teacher and inspired me to include and use humor in my teaching.

My committee members have helped me navigate the hurdles that I faced in my research project. Dr. Todd K. Keylock, our exercise and wound healing expert has been involved throughout the project and has been always willing to share his knowledge of techniques and experimental equipment. Dr. Howard C. Cromwell helped me figure out the statistics and bettering the final write up. Dr. Scott O. Rogers has provided crucial feedback throughout the project. Dr. Vipaporn Phuntumart has been gracious enough to be a committee member towards the end of the project and provided calm support. Dr. Carmen Fioravanti was part of the initial committee and played an essential role in the project before he retired.

I am extremely grateful to Dr. Jeff Miner for providing funding for the project at a critical point. All the faculty in the Biological Sciences department have been very helpful and supportive during my time here. Lorraine, DeeDee and Denise from the Biology office; Linda, Chris, Steve and Susan from the stockroom; Matt, Mike and Rob from the animal facility; Sheila from the autoclave room have always been kind and accommodating to all the research and academic requests, which I really appreciate. Dr. Pavel Anzenbacher from the Chemistry department and his student Elena helped me run the plate reader for protein assay and I am thankful for that. Dr. Stanislaw Stepkowski from The University of Toledo and his student Dulat; Dr. Gary Ross from
Bio-Rad helped me run the cytokine assay and I can’t thank them enough for that. Special thanks to all the undergraduate students who were involved in various aspects, throughout this project.

My parents have always been there for me and have always encouraged me to do my best. My family back in India and our extended family of friends in U.S. have helped us in thick and thin. This has been a very tough and long journey and I couldn’t have accomplished it without the unconditional love, support and encouragement of my lovely wife, Kanchan. I can’t thank her enough for all her sacrifices and for our adorable daughter, Anika who is the love of our life.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Significance</td>
<td>1</td>
</tr>
<tr>
<td>Specific Aims</td>
<td>2</td>
</tr>
<tr>
<td>Background</td>
<td>4</td>
</tr>
<tr>
<td>CHAPTER I: EFFECT OF PCB AND EXERCISE ON WOUND HEALING BY</td>
<td></td>
</tr>
<tr>
<td>MEASURING WOUND SIZE</td>
<td>11</td>
</tr>
<tr>
<td>Introduction</td>
<td>11</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>13</td>
</tr>
<tr>
<td>Animal Care</td>
<td>13</td>
</tr>
<tr>
<td>PCB Exposure</td>
<td>13</td>
</tr>
<tr>
<td>Exercise Regimen</td>
<td>13</td>
</tr>
<tr>
<td>Wound Creation and Wound Size Measurement</td>
<td>14</td>
</tr>
<tr>
<td>Euthanasia</td>
<td>15</td>
</tr>
<tr>
<td>Statistics</td>
<td>15</td>
</tr>
<tr>
<td>Results</td>
<td>16</td>
</tr>
<tr>
<td>Wound Size Changes</td>
<td>16</td>
</tr>
<tr>
<td>Effect of Exercise on Wound Healing</td>
<td>18</td>
</tr>
<tr>
<td>Effect of PCB on Wound Healing in Not Exercised Animals</td>
<td>18</td>
</tr>
<tr>
<td>Effect of PCB on Wound Healing in Exercised Animals</td>
<td>18</td>
</tr>
<tr>
<td>Discussion</td>
<td>22</td>
</tr>
</tbody>
</table>
CHAPTER II: EFFECT OF PCB AND EXERCISE ON CYTOKINE

CONCENTRATIONS IN WOUND TISSUE

Introduction
Materials and Methods
Wound Cytokine Analysis
Tissue Extraction Procedure
Protein Assays
Cytokine Analysis
Statistics
Results

Effect of Exercise and PCB (Aroclor 1254) on IL-1β levels
Effect of Exercise and PCB (Aroclor 1254) on IL-6 levels
Effect of Exercise and PCB (Aroclor 1254) on KC levels
Effect of Exercise and PCB (Aroclor 1254) on MCP-1 levels
Effect of Exercise and PCB (Aroclor 1254) on TNF-α levels

Discussion

CHAPTER III: EFFECT OF PCB AND EXERCISE ON AVERAGE BODY TEMPERATURE FOLLOWING LPS STIMULATION

Introduction
Materials and Methods
Euthanasia
Lipopolysaccharide Administration
Average Body Temperature Measurement
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Comparison of the Wound Size in Animals from Day 0 (day of wound creation) to Day 9</td>
</tr>
<tr>
<td>2</td>
<td>Effect of Exercise on Wound Size in Mice Administered Varying Doses of PCB</td>
</tr>
<tr>
<td>3</td>
<td>Comparison of Wound Sizes on Different Days in Animals Not Exercised and Administered Varying Doses of PCB</td>
</tr>
<tr>
<td>4</td>
<td>Comparison of Wound Sizes on Different Days in Exercised Animals Administered Varying Doses of PCB</td>
</tr>
<tr>
<td>5</td>
<td>Effect of Exercise and PCB (Aroclor 1254) on Levels of IL-1β in Wound Tissue at Day 3 and Day 5 Post Wounding</td>
</tr>
<tr>
<td>6</td>
<td>Effect of Exercise and PCB (Aroclor 1254) on levels of IL-6 in Wound Tissue at Day 3 and Day 5 Post Wounding</td>
</tr>
<tr>
<td>7</td>
<td>Effect of Exercise and PCB (Aroclor 1254) on Levels of KC in Wound Tissue at Day 3 and Day 5 Post Wounding</td>
</tr>
<tr>
<td>8</td>
<td>Effect of Exercise and PCB (Aroclor 1254) on Levels of MCP-1 in Wound Tissue at Day 3 and Day 5 Post Wounding</td>
</tr>
<tr>
<td>9</td>
<td>Effect of Exercise and PCB (Aroclor 1254) on Levels of TNF-α in Wound Tissue at Day 3 and Day 5 Post Wounding</td>
</tr>
<tr>
<td>10</td>
<td>Comparison of Changes in Average Body Temperatures Post LPS Administration in Animals from Day 0 (Day of PCB Administration) to Day 8</td>
</tr>
<tr>
<td>11</td>
<td>Effect of Exercise on Changes in Average Body Temperatures after LPS Administration in Mice</td>
</tr>
</tbody>
</table>
12 Comparison of Changes in Average Body Temperatures after LPS Administration in Animals Not Exercised and Administered Varying Doses of PCB ........................ 51
13 Comparison of Changes in Average Body Temperatures after LPS Administration in Exercised Animals Administered Varying Doses of PCB............................... 52
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Detailed distribution of the mice used for wound healing study</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>Detailed distribution of the mice for cytokine analysis study</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>Detailed distribution of the mice for LPS stimulation study</td>
<td>46</td>
</tr>
</tbody>
</table>
INTRODUCTION

Significance

Polychlorinated biphenyls (PCB) are environmental contaminants that were manufactured and used in large quantities for over 40 years because of their wide range of applications. However, eventually the harmful effects of these chemicals were observed and their commercial production and use was banned (Shields, 2006). By the time PCB manufacture was halted a considerable amount had already been released into the environment, and since they are very slowly degraded, is still present there in large quantities (Hopf et. al., 2009). PCB are known endocrine disruptors and also have been shown to cause carcinogenecity, genotoxicity, reproductive toxicity and immunological effects (Safe, 2004; Meserve et al., 1992; Pruitt et al., 1999). Many studies have shown that PCB exposure leads to immunosuppression in mice and humans, however some studies have shown an increase in immunological response (Heilmann et al., 2006; Schell et al., 2009; Harper et al., 1995). Aroclor 1254 which is used in the present study is a mixture of PCB congeners with 54% chlorination and was widely manufactured and released into the environment (Segre et al., 2002). The changes in immunological response that have been reported can be associated with longer wound healing time, since alterations in inflammatory phase of wound healing can adversely affect wound healing. This aspect of PCB exposure affecting wound healing has not been studied previously. Similarly, PCB induced immunological response changes can also affect the systemic immune response, which will be studied using lipopolysaccharide (LPS) stimulation.

Exercise has been shown to have various beneficial physiological effects as well as reducing the harmful effects of various environmental and biological factors including stress, exposure to certain toxins and infection (Woods et al., 2002; Senchina, 2009; Batista et al.,
2007). Studies have found that pre-exposure to exercise can alter the local and systemic molecular markers of the immune response, there is additional evidence that wound healing can be promoted with pre-exposure to exercise (Keylock et al., 2007; Spence et al., 2009).

The complexity of the effect of substances like PCB can be studied in a detailed manner only in multicellular animals. Also immune functions, activity, modification and the effect of exercise can be studied in great detail in lab animals that can perform physical activity which is similar to exercise in humans. Mice were chosen for the present study as they fulfilled the above criteria and also, since they are monogastric (single stomach) and omnivorous (consume various kinds of food), the findings can be generalized to other animals including humans, especially because we are dealing with the immune system. Mice also were chosen for this study as they have been widely used in immunological studies and as the findings in them can be closely correlated with human immunological studies. Also a recent study reported that the effects of PCB on various systems including the immune system are similar in mice and humans (Yoshizawa et al., 2007). Another recent investigation has used mice as the study animal to evaluate the effect of exercise on wound healing. C57BL/6 mice were used as these were the mice that were used in a previous study that examined the effects of PCB on immune function and also administered PCB and LPS by intraperitoneal (i.p.) injections, as used in this study (Zhao et al., 1997).

**Specific Aims:**

**Aim I (Chapter I):** To study the effect of PCB exposure on local wound healing and determine whether exercise can alter these effects.

**Hypothesis:** It is anticipated that PCB exposure will cause modulation of the immune response and subsequently prolong wound healing times. Exercise has been shown to reduce wound
healing times and the hypothesis is that in PCB exposed animals, exercise would be able to reduce wound healing times when compared to the non-exercised group.

**Plan:** Wound healing will be monitored by comparing the wound sizes as they heal and also by analysis of the concentration of cytokines IL-1β, IL-6, (KC), (MCP-1), and (TNF-α) from the wound tissue as it heals.

**Aim II (Chapter II):** To study the effect of PCB exposure on cytokine concentrations in wound tissue and determine if exercise can alter these effects.

**Hypothesis:** It is anticipated that PCB exposure will alter the levels of cytokines in the wound tissue and subsequently lengthen wound healing times. Exercise has been demonstrated to reduce the levels of cytokines in wound tissue and the hypothesis is that in PCB exposed animals, exercise would be able to reduce wound healing times through reduction of cytokine amounts.

**Plan:** Concentrations of cytokines IL-1β, IL-6, (KC), (MCP-1), and (TNF-α) will be measured from the wound tissue using a Bio-Plex Pro Mouse Cytokine 23-plex Assay kit.

**Aim III (Chapter III):** To study the effect of PCB exposure followed by LPS exposure on systemic immune response and evaluate if exercise can alter these effects.

**Hypothesis:** Previous research had shown that PCB exposure followed by exposure to LPS impairs the systemic immune response to LPS. Considering that exercise has a beneficial effect on the systemic immune response by reducing inflammation, the hypothesis is that in PCB treated and LPS exposed animals, exercise can alter the systemic immune response when compared to the non-exercise group.

**Plan:** The systemic effects of exercise on immune function in PCB exposed animals will be examined by exposure to lipopolysaccharide (LPS, administered by intraperitoneal injections) and then the animals were pictured using an infrared camera post injection with PCB and the
pictures were analyzed using the accompanying software to determine average body temperatures.

This novel project investigates how a combination of exercise and PCB (polychlorinated biphenyl) exposure influence the rate at which skin wounds (inflicted by punch biopsy) heal and also what effects exercise, PCB and the combination has on systemic inflammation. Examining this combination was interesting for two main reasons. The first reason stems from previous research findings that exercise results in wounds healing at a faster rate because it stimulates the immune system. On the other hand, exposure to PCB (intraperitoneal injection) alters the immune system, in some cases by stimulation and in others by inhibition. So, it would be of interest to determine if PCB delays wound healing and if moderate intensity exercise (5 days a week for 3 weeks) returns wound healing time to normal in PCB exposed mice. The second reason is that there is PCB in Great Lakes fish at a concentration that can cause health problems in people who regularly eat the fish harvested from that fresh water source. If exercise restores wound healing rate in PCB exposed mice, it might also reverse that and other health problems in people eating Great Lakes fish.

**Background**

PCB mass production started in the 1930s and its insulating and non-flammable properties made it a very useful product in the energy industry. Other applications for PCB were discovered and they found their way into inks, adhesives and pesticides (Gladen *et al.*, 1988; Jacobson and Jacobson, 1997). Soon some of the harmful effects of exposure to PCB in the form of chloracne were observed in workers in PCB manufacturing facilities. Chronic exposure also led to skin, respiratory and liver problems. However, the bigger risks of PCB were uncovered by two accidental exposure in Fukoka and Nagasaki, Japan, 1968 and Taiwan in 1978 where
contamination of rice bran oil led to chloracne, liver and neurological disorders in the exposed population. Exposed pregnant women delivered children with developmental and neurological deficits and their hair and nails were discolored (Guo et al., 1999; Tsukimori et al., 2012)). Discovery of all these health hazards of PCB exposure established them as a toxin and its eventual worldwide ban in the 1970s.

During the 40 years when PCB production was at its peak approximately 1.5 million tons of PCB were produced. Since they were widely produced and initially were not known to be a health hazard, PCB disposal was not regulated by either industries or consumers. The disposal of PCB into the water bodies by the industries and water run off from farms using pesticides with PCB led to environmental contamination (Yadetie et al., 2017; Hileman, 1993). Even now many older buildings have insulators, capacitors and generators that contain PCB. The Twin Towers in New York had generators which contained PCB, that was released into the environment when the towers were destroyed (Litten et al., 2003; Butt et al., 2004). Caution is necessary in handling and disposal of any equipment that contains PCB, so that they are not released into the environment. Though current release of PCB into the environment has nearly ceased, the large amounts that already exist possess a health risk because of the persistence of this material.

PCB is a biphenyl with two connected benzene rings that have varying number of chlorine atoms. There are 209 congeners of PCB as a result of variability in number and positioning of the chlorine atoms. The greater the number of chlorine atoms, the more they are lipophilic and have longer half life and subsequently more toxicity (Bodin et al., 2007; McKinney and Waller, 1994; Tan et al., 2004). Presence of two or more chlorine atoms in the ortho position makes PCB non-coplanar and their absence at that position makes them coplanar. The toxicity and mechanism of action of PCB varies on whether they are coplanar or
non-coplanar. The coplanar PCB are dioxin-like in their effects, mainly acting via the aryl hydrocarbon receptor (Ah), whereas the non-coplanar PCB have been shown through a variety of pathways that are still not completely understood (Tilson et al., 1998). PCB are available commercially as mixtures like aroclor 1254 (A1254), A1242 and A1260, where the first two numbers stand for the numbers of carbon atoms and the last two numbers stand for the percentage of chlorination. For example, A1254 (used in the current study) has 12 carbon atoms in each biphenyl ring and has 54% chlorination as a mixture. These mixtures contain both coplanar and non coplanar PCB congeners (Fikslin and Santoro, 2003). This is very similar to what would be found in the environment and that is why these mixtures have been widely used in animal and in vitro studies. Animal studies have administered PCB in varying doses through diet, oral gavage and injections both subcutaneous and intra peritoneal (i.p., similar to the current study) (Arena et al., 2003; Tang et al., 2015; Aly et al., 2009).

PCB that have made their way into the water bodies settle at the bottom and are consumed by diatoms, incorporated in their fat deposits, and slowly make their way up the food chain. Since PCB are lipophilic, they tend to bioaccumulate in the fatty tissue and humans consuming larger fish from these bodies of water are exposed to high levels of PCB. A study of fish in different bodies of water in the U.S. has shown elevated PCB levels in fish (Stahl et al., 2009). In the human body they also accumulate in the adipose tissue in the breast, visceral fat, liver and brain, continuing to have harmful effects (Brasher and Wolff 2004; Monikh et al., 2014). Transplacental and maternal milk transfer of PCB from mother to child and its harmful effect on development in child has been extensively studied and is a cause of concern in pregnant or nursing women consuming large quantities of fish. This developmental exposure in children
has been shown to have long term implications on the endocrine, immune and neurological development of these offsprings (Ulbrich and Stahlman, 2004).

Various in vitro, animal, and human studies have demonstrated that PCB affect various body systems. PCB are known endocrine disruptors, affecting thyroid, sex, and adrenal hormones, further leading to developmental defects, infertility and neurological problems. Gastointestinal issues including nausea, loss of appetite and anorexia have been reported with chronic PCB exposure (Donahue et al., 2002; Gaum et al., 2016; Quinete et al., 2014; Provost et al., 1999; James et al., 1993).

PCB have been shown to have various effects on the immune systems mainly immunotoxic and immunosuppressive. Studies in animals have also shown that PCB exposure can lead to certain types of cancer. Follow up studies in people exposed to contaminated rice bran oil in Japan have revealed higher mortality from liver and lung cancers (Onozuka et al., 2009). Chronic and high levels of exposure to PCB has also been associated with presence of anti nuclear and anti thyroid antibodies (Crinnion, 2011). Increased susceptibility to chicken-pox and middle ear infections have also been associated with PCB exposure in Dutch pre school children (Weisglas-Kuperus et al., 2000; Weisglas-Kuperus et al., 2004). On the cellular levels PCB have been shown to decrease survival of monocytes, thymocytes and splenocytes (Tan et al., 2003; Ferrante et al., 2011; Shin et al., 2000; Yoo et al., 1997). PCB has also been shown to hamper the oxidative burst of neutrophils (Narayanan et al., 1998). PCB have also been shown to reduce the levels of cytotoxic t-cells (Fournier et al., 2000). A wound healing study in earthworms revealed that wound healing is slower in PCB administered earthworms in comparison to the earthworms not administered PCB (Cooper and Roch, 1992).
Immune system is the defense mechanism of the body against foreign material and injury. Immune system can be broadly classified into innate and adaptive immunity. They are not totally separate and show a lot of overlap. Innate immunity is not specific to the invading organism and act as the first line of defense. Neutrophils, macrophages and dendritic cells are some of the major cells involved in this process. Various cytokines and chemokines released by these cells are responsible for recruitment of immune cells part of both innate and adaptive immunity. There is no memory component associated with innate immune response. On the other hand, adaptive immunity is more specific to the invading organism and involves T and B lymphocytes. Adaptive immunity is further classified into cell mediated immunity (T lymphocytes) and humoral (antibody mediated) immunity (B lymphocytes). Both cell mediated and humoral immunity produces memory cells that help develop more rapid responses with subsequent exposure. B lymphocytes secrete antibodies that are very specific for the targeted microorganism (Getz, 2005; Portou et al., 2015; Nikolich-Žugich and Davies, 2017). Immune response can be studied at a local level or systemic level. In this study, we have used creation of a skin wound to study the local immune response and administration of LPS to study systemic immune response. Both these models have been previously used for studying the respective immune responses in animals and humans (Ahmed and Antonsen, 2016; Werner and Grose, 2003; Rodewohl et al., 2017).

It has been widely held that regular and moderate exercise has many beneficial health effects. Exercise and its effects have been studied by clinicians for years in relation to the rate of progression of various infections (Brenner et al., 1994). It is only over the last few decades that the molecular mechanisms of exercise on health and immune system have been studied in detail. Researchers are slowly coming to a consensus that physical activity producing a U-shaped
resistance curve, with both no and extreme physical activity having a deleterious effect, whereas moderate physical activity has beneficial effect on both overall health and immune system function (Merghani et al., 2016). Moderate intensity exercise has been shown to have beneficial effects, retarding development of various chronic conditions like cardiovascular disease, atherosclerosis and chronic inflammation and it also reduces the progression of these diseases and certain neoplasms if they are already present (Mathur and Pedersen, 2008; Goh and Ladiges, 2014). The presence of more anti-inflammatory cytokines because of exercise has been demonstrated and this is essential for the positive effects of exercise (Gleeson et al., 2011). The effect of exercise has also been studied in older mice where the immune function is suboptimal and this results in greater susceptibility to infection and delayed recovery from them. These animals have also been found to have delayed wound healing. Moderate intensity exercise in these animals improved wound healing rates. It also reduced the levels of pro-inflammatory cytokines in the wound tissue of these animals (Keylock et al., 2008).

Studies on amounts of immunoglobulin A (IgA) secreted in the saliva have shown that concentrations of IgA are reduced by chronic extreme exercise like that seen in athletes, whereas the concentrations are slightly increased with exercise of moderate intensity. This is suggested as one of the reasons why upper respiratory tract infections are common in athletes after a competitive event. (Peake et al., 2016). High intensity exercise has been shown to reduce the neutrophil burst capacity and thus make the individual more susceptible to infections (Davison and Jones, 2015). However, moderate intensity exercise has been shown to reduce infiltration of neutrophils at areas of chronic inflammation (Kawanishi et al., 2015)). Similarly, reduction in macrophage infiltration is also seen with regular moderate intensity exercise (Trott et al., 2016). This is beneficial in these cases of chronic inflammation but could be detrimental in resolution of
chronic infection. The effects of exercise of varying intensities on the levels of dendritic cells and natural killer cells is not clear. The numbers of T and B lymphocytes are not altered by moderate intensity exercise. However, prolonged intense exercise as seen in athletes tends to reduce the number of the T and B lymphocytes and that could be one of the reasons why these individuals are more prone to infections (Simpson et al., 2015).

Considering the evidence that PCB have immunotoxic effect, the present study examines the effect of PCB on the local immune response (wound healing) and systemic immune response (LPS stimulation) and evaluates how moderate intensity exercise can reverse some of the harmful effects of PCB.
CHAPTER I: EFFECT OF PCB AND EXERCISE ON WOUND HEALING BY MEASURING WOUND SIZE

Introduction

Wound healing is a complex process that involves 4 phases: hemostasis, inflammation, proliferation and remodeling that overlap considerably. Hemostasis is the first stage where the primary role is to stop the bleeding which involves constriction of the blood vessels and formation of a clot. The injured tissue releases cytokines and growth factors that are essential for recruitment of the immune cells involved in the next phase. The inflammation phase mainly involves neutrophils and the macrophages. Neutrophils clear the wound area of any microorganisms and dead tissue. One of many roles of macrophages is to secrete the pro-inflammatory cytokines that like IL-1, IL-6 and TNF-α, that are further involved in recruiting monocytes and T-cells to the tissue (which play a role in the inflammation and proliferation phases). Macrophages also limit the inflammatory phase by phagocytosis of the apoptotic neutrophils, which transforms macrophages to produce more anti-inflammatory cytokines by a process called efferocytosis. The secretion of cytokines and growth factors by the macrophages plays a major role in initiating the next two phases of wound healing. Thus, macrophages play a central role in wound healing.

The next phases of proliferation and remodeling involve recruitment of endothelial cells, also known as keratinocytes and fibroblasts, along with revascularization. Both these cells on migration generate a collagen network which is essential in formation of the extracellular matrix. Cells called myofibroblasts are responsible for contraction of the wound. This sequence of events is typical of wound healing in humans. However certain differences in mice, like lack of dermal papillae and sweat glands, may alter the wound healing process in these animals, where wound contraction constitutes their primary means of healing. These differences are very important
when comparing wound healing between different model systems. In humans wound healing is affected by both local factors like infection and oxygenation and systemic factors like ageing, smoking, alcohol, stress, diabetes and obesity (Guo and DiPietro, 2010; Pence and Woods, 2014).

As noted above, inflammation involves the recruitment of various immune cells and secretion of various cytokines that not only help in preventing infection, but also play an essential role in reepithelization of the wound tissue. PCB are shown to have immunotoxic effect and impair the functioning of several immune cells (Loose et al., 1981; Tryphonas et al., 1991). Previous studies have shown that PCB can hamper wound healing (Roch and Cooper, 1991; Ville et al., 1995). However, these earlier studies were done in earthworms where the PCB was absorbed through the skin and the wound sizes were measured 24 hours after wound creation. In the present study, the aim was to determine the effect of PCB in varying doses on wound healing in mice, where wound sizes were monitored for a longer time (9 days rather than 1 day). In the direction, opposite to PCB, exercise has been shown to have beneficial effects on the immune system. Moderate intensity exercise has been shown to improve healing after cardiac surgery, prevent further worsening of atherosclerosis and cancer progression through its effect on the immune cells (Mathur and Pedersen, 2008; Goh and Ladiges, 2014). The importance of appropriate exercise in reducing the risk of chronic disease or contribute to increasing quality of life has been the subject of various studies (Pedersen and Saltin, 2006; Warren et al., 2010). However, strenuous exercise can have detrimental effects on the immune system (Shek and Shephard, 1998). Moderate intensity exercise led to more rapid wound healing rates in aged mice and reduced the levels of pro-inflammatory cytokines in wound tissue (Zhang et al., 2016; Keylock et al., 2008). The aim of this part of the present study is to determine if PCB
administration negatively effects wound healing and if exercise can reduce some of those negative effects and improve wound healing.

**Materials and Methods**

**Animal Care**

Female C57BL/6 mice (8 weeks of age) were obtained from Harlan Laboratories, Indianapolis, IN and housed in the animal facility. All the animal studies were conducted as approved by the (Bowling Green State University) BGSU Institutional Animal Care and Use Committee (IACUC) under protocol no. 10-013. The mice were individually housed in shoe box cages and maintained on a reverse light-dark cycle. They were provided food (Teklad Mouse Breeder Diet 8626, Envigo, Madison, WI) and water ad libitum, and their body weights were recorded daily. At the end of testing, animals were euthanized by rapid CO₂ inhalation.

**PCB Exposure**

All the animals were given 2 weeks to acclimatize to the animal facility before the study began. All the animals in the present study (both not exercised & exercised groups) were administered PCB (Aroclor 1254; Accustandard Inc. New Haven CT, USA) dissolved in corn oil via intraperitoneal injection at a volume of 10 µl/g body weight. PCB doses administered were 0 (PCB 0), 100 (PCB 100), 500 (PCB 500) or 1000 (PCB 1000) µg/g. Following acclimatization, the animals in the group not exercised were housed in the animal facility for 3 weeks without exercise and PCB injection was administered 2 days after this 3-week period without exercise was over. Following acclimatization, the animals in the exercised group began exercise for a 3-week period, 2 days after completion of the exercise period they were administered PCB.
**Exercise Regimen**

The animals not exercised remained in cages without exercise during the 3-week period. The carts holding the cages of these animals were tethered to the table with the motorized treadmill using big metal clamps, so that these no exercise animals not exercised were exposed to the same sounds and vibrations as the animals in the exercise group. The animals in the exercised group exercised by running on a motorized treadmill (Jog-A-Dog model DC6 1H.P.) which was adapted with individual lanes for mice. This running was done for 30 minutes daily, five days a week for 3 weeks and consisted of running at 10-12 m/min with a 6% incline (considered moderate intensity). These exercise regimens were carried out at the beginning of the active period of these animals (0700-0900 as they are on a reverse light-dark cycle). Previous studies have shown that this intensity of exercise is effective in altering immune function (Keylock et al., 2008).

**Wound Creation and Wound Size Measurement**

This was carried out in the animals used in Aim I (Table 1) of the study. Wounds were made two days following PCB exposure. Mice were anesthetized with isoflurane ( Isoflo®) in 100 % oxygen at a flow rate of 2-3 L/min by cone mask during wound creation. Mice were then administered 0.05 µg/g Buprenex (Buprenorphine hydrochloride, 0.3 mg/ml, Reckitt Benckiser, Healthcare (UK) Ltd., Hull, England). A similar dose of Buprenex was administered twice a day for 2 days post wounding. Wahl Peanut Hair Trimmer (Sterling, IL) was used without guides to remove the hair over an area on the upper back, a location inaccessible to the animal for the creation of wounds. The shaved area was then cleaned with Betadine and 70% ethanol. Wounds were made using a 3.5 mm sterile disposable punch biopsy instrument (Robbins Instruments, Chatham, NJ) to create one full thickness dermal punch resulting in two wounds. A Canon EOS
Rebel XTi camera was used to photograph the wound at the same time daily until the wounds were 10% of original size or for two weeks after the wounds were made, whichever came first. Image J software (version 1.41o, NIH) was used to analyze the wound and compare to the reference wound, thus allowing comparison between rates of wound healing.

**Table 1: Detailed distribution of the mice used for wound healing study**

<table>
<thead>
<tr>
<th>PCB doses (µg/g)</th>
<th>No Exercise</th>
<th>Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound Healing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>100</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>500</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>1000</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

**Euthanasia**

The animals were euthanized by rapid CO₂ asphyxiation after the wound size reached 10% of the original wound or two weeks post wounding, whichever came first. A 6.0 mm punch biopsy instrument was used to harvest the wound and the surrounding tissue. Organ weights, including those of the spleen and thymus gland, were determined.

**Statistics**

Statistical analysis was done using SPSS software (IBM, 2015, version 23). For the wound size data, general linear model Repeated Measures ANOVA (along with post hoc tests, Bonferroni and Tukey) was performed for PCB, Exercise and Day. Wound size on Day 0 (day of wound creation) over all the groups was normalized to 1 and all the values for the following days were sizes compared to Day 0. Error bars are +/- 1 standard error of the mean (SEM). Significance was determined as p ≤ 0.05.
Results

Repeated Measures ANOVA yielded no significant effects or trends. Non parametric tests were run only to observe the day effect on wound size among all groups combined (Fig. 1). For the day effect, related samples Friedman’s two way analysis of variance by ranks were run.

Wound Size Changes

Wound size was measured starting from Day 0 (date of wound creation) to the point where either a scab had formed or wound size was reduced to 10% of original (one or the other of these events had occurred by Day 9 in all mice). All the treatment groups were combined and only the day effect was examined using Friedman’s Two-Way ANOVA \( (F_{6} = 223.066; p < 0.001) \) which yielded the following significant results. Wound sizes decrease consistently over the course of the days of observation (Fig. 1). The wounds were progressively smaller on Day 2 \( (\ast: W = 1.762; p = 0.004) \), Day 3 \( (\ast: W = 2.357; p < 0.001) \), Day 4 \( (\ast: W = 3.440; p < 0.001) \), Day 5 \( (\ast: W = 4.560; p < 0.001) \) and Day 6 \( (\ast: W = 5.464; p < 0.001) \) as compared to Day 0. Day 3 \( (\#: W = 1.857; p = 0.002) \), Day 4 \( (\#: W = 2.940; p < 0.001) \), Day 5 \( (\#: W = 4.060; p < 0.001) \) and Day 6 \( (\#: W = 4.964; p < 0.001) \) wound sizes were smaller as compared to Day 1. Also, as compared to Day 2, wound sizes were smaller on Day 4 \( (@: W = 1.679; p = 0.008) \), Day 5 \( (@: W = 2.798; p < 0.001) \) and Day 6 \( (@: W = 3.702; p < 0.001) \), and wound sizes on Day 5 \( (@: W = 2.202; p < 0.001) \) and Day 6 \( (@: W = 3.107; p < 0.001) \) were smaller as compared to Day 3. Wound sizes also were smaller on Day 6 \( (\$: W = 2.024; p < 0.001) \) as compared to Day 4.
Fig 1: Comparison of the Wound Size in Animals from Day 0 (day of wound creation) to Day 9 (n = 11-49): Animals from all the treatment groups were combined and used to study the day effect. The data has been normalized with the initial wound size on the day of production (Day 0) being 1. As can be expected, the wound size decreases consistently over the period, reduced to less than 10% in 9 days. Wound size is significantly lower on all the previous days except for the immediate day before. For example, wound size on Day 3 is significantly lower than Day 0 and Day 1, but not Day 2. All the other days follow a similar pattern.
Effect of Exercise on Wound Healing in Mice Treated with no PCB

In mice administered no PCB, no significant effect or trends were observed in wound sizes between exercised and not exercised mice. However, comparison of the figures illustrates a pattern, where wound sizes decrease at a greater rate in exercised mice administered no PCB (Fig: 2A)

Effect of Exercise on Wound Healing in Mice Treated with Varying Doses of PCB

In mice administered varying doses of PCB, no significant effect or trends were observed in wound sizes between exercised and not exercised mice. However, comparison of the figures illustrates a pattern, where wound sizes decrease at a greater rate in exercised mice administered PCB 500 (Fig: 2C). However, in PCB 100 mice (Fig: 2B), mice not exercised healed at a greater rate. Whereas, wound healing rates appear very similar in exercised and not exercised mice administered PCB 1000 (Fig: 2D).

Effect of Varying Doses of PCB on Wound Healing in Not Exercised Animals

Animals that were not exercised did not show any significant changes or trends in wound healing rates with varying PCB doses. Observation of the graphs, shows a pattern where the rates of wound healing are faster in PCB 1000 administered animals as compared to PCB 0 (Fig. 3C), PCB 100 (Fig. 3E) and PCB 500 (Fig. 3F). Comparison of the other PCB doses did not yield any obvious pattern (Fig. 3A, B, D).

Effect of Varying Doses of PCB on Wound Healing in Exercised Animals

No significant changes or trends in wound healing rates with varying PCB doses were observed in animals that were exercised. However, the figures reveal a pattern where the rates of wound healing are less in PCB 100 administered animals as compared to PCB 0 (Fig. 4A),
Fig 2: Effect of Exercise on Wound Size in Mice Administered Varying Doses of PCB (n = 4-10): A: Animals that were administered PCB in varying doses did not show any significant differences or trends in wound size on different days after wound production, irrespective of whether they were exercised or not. However, observation of the graph shows that there seems to be a difference in the mean wound sizes Day 1 through Day 6 between the exercise and no exercise groups with all doses of PCB, except PCB 1000.
Fig 3: Comparison of Wound Sizes on Different Days in Animals Not Exercised and Administered Varying Doses of PCB (n = 4-10): C, E and F: The mean wound size is lower on Day 2 through Day 6 in PCB 1000 treated animals as compared to PCB 0, PCB 100 and PCB 500 administered mice, though no significant differences or trends were observed. A, B, and D: No significant differences or trends and no difference in means were observed when the other doses of PCB were compared.
Fig 4: Comparison of Wound Sizes on Different Days in Exercised Animals Administered Varying Doses of PCB (n = 4-10):

A, D and E: The mean wound size is higher on Day 1 through Day 6 in PCB 100 treated animals as compared to PCB 0, PCB 500 and PCB 1000 administered mice, though no significant differences or trends were observed. B, C, and F: No significant differences or trends and no difference in means were observed when the other doses of PCB were compared.
PCB 500 (Fig. 4D) and PCB 1000 (Fig. 4E). No particular pattern was observed with other PCB doses (Fig. 4B, C, F).

**Discussion**

There are many factors that affect wound healing. One of the important phases of wound healing is the inflammatory phase and any modulation of this could affect wound healing rates (Guo and DiPietro, 2010). Wound healing rates have been shown to be greater in aged mice that were exercised as compared to sedentary mice, but no statistically significant differences were visible in younger mice (Keylock *et al.*, 2008). Wound healing data from the present study which uses young adult mice shows a pattern of faster wound healing in exercised animals as compared to the mice not exercised. This pattern was observed in mice either dosed with no PCB and PCB at 500 µg/g. PCB administered at 100 µg/g resulted in a reversed pattern of slower wound healing in exercised mice. Exercise does not appear to alter rate of wound healing rates in mice given 1000 µg/g of PCB. Wound healing in earthworms has been shown to be impaired by PCB administration, 24 hours after wound creation (Roch and Cooper, 1991; Ville *et al.*, 1995). Additionally, PCB have been demonstrated to impair the survival and function of macrophages (Ferrante *et al.*, 2011). There is research evidence that absence or reduced concentration of macrophages increases rate of wound healing (Martin and Leibovich, 2005). It is possible that this effect on macrophages is only seen at greater doses of PCB, 1000 µg/g in the present study. A macrophage-mediated effect could also be the reason why PCB administered at 1000 µg/g caused a pattern of most rapid wound healing in animals not exercised as compared to all other PCB doses. In the exercised animals wound healing occurred less rapidly in the animals given PCB at 100 µg/g as compared to the animals administered other PCB doses. PCB have been shown to activate lysosomal activity and increase production of reactive oxygen species by
neutrophils, that appears to be a compensatory mechanism to its inhibition of macrophages (Ganey et al., 1993). This compensation is likely accentuated in exercised mice with lesser amounts of PCB, 100 µg/g in this study. Cytokines play an important role in the inflammatory phase of wound healing (Menke et al., 2007; Edwards and Harding, 2004). The second segment of the present study focused on determination of tissue concentrations of cytokines obtained from wound tissue on Day 3 and Day 5 of wound healing. These days were chosen for cytokine concentration measurements because the rates of wound healing differed around these days in the first segment of the present study and there are previous data for cytokines in wound tissue on these days (Keylock et al., 2008).
CHAPTER II: EFFECT OF PCB AND EXERCISE ON CYTOKINE CONCENTRATIONS IN WOUND TISSUE

Introduction

The primary aim of this part of the study was to measure the concentrations of various cytokines in wound tissue of mice exercised or not exercised, with or without PCB exposure. Various immune cells and the cytokines released by them play an important part in the inflammatory phase of wound healing by not only recruiting other immune cells, but also in reepithelization (Barrientos et al., 2008). Previous studies have shown that excessive inflammation with the presence of increased pro-inflammatory cytokines can delay wound healing, especially in aged animals (Swift et al., 2001; Ashcroft et al., 2002). On the other hand, mice without macrophages and neutrophils experience greater rates of wound healing (Martin and Leibovich, 2005). It has been shown that exercise improves wound healing rate in obese and aged mice (Pence and Woods, 2014). The concentrations of pro-inflammatory cytokines like TNF-α, KC and MCP-1 have been observed to decrease in aged mice after exercise and these findings correlated with more rapid wound healing rates in these animals. However, this study did not find significant positive effects of exercise on wound healing in younger mice. In an evolutionarily less complex species, PCB administration in earthworms has impaired wound healing and increased allograft rejection (Roch and Cooper, 1991; Ville et al., 1995). Additionally, in vitro studies have found PCB to cause enhanced stimulation of neutrophils to produce reactive oxygen species (Ganey et al., 1993). However, the function of human and mouse macrophages and mouse splenocytes has been shown to be impaired by PCB administration (Ferrante et al., 2011; Shin et al., 2000; Yoo et al., 1997). No previous studies have determined the amounts of cytokines in wound tissue of animals administered PCB. The
cytokine data will give us a novel insight into whether exercise can modulate PCB induced modification of cytokine concentrations and thus negating some of the immunotoxic effects of PCB.

**Materials and Methods**

Animal care, PCB exposure, exercise regimen, wound creation and euthanasia were identical to those of Chapter I, except that mice were euthanized on Day 3 or Day 5 post-wounding

**Wound Cytokine Analysis**

IL-1, IL-6, keratinocyte chemoattractant (KC), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-α (TNF-α) protein concentrations in wound tissue were determined using Bio-Plex Pro Mouse Cytokine 23-plex Assay kit (Bio-Rad Laboratories, Inc., Philadelphia, PA).

**Table 2: Detailed distribution of the mice for cytokine analysis study**

<table>
<thead>
<tr>
<th>PCB doses (µg/g)</th>
<th>Day 3</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Exercise</td>
<td>Exercise</td>
</tr>
<tr>
<td>0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>100</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>500</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>1000</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>
Tissue Extraction Procedure

The wound and the surrounding tissue from animals that were euthanized by CO₂ asphyxiation at Day 3 and Day 5 after wound creation were harvested using a 6mm punch biopsy instrument (Robbins Instruments, Chatham, NJ). The tissue was flash frozen in liquid nitrogen and then stored in -80°C freezer. This tissue was homogenized using a protocol used by Frank and Kampfer (2003). The wound tissue was homogenized in an extraction solution containing sterile 1X PBS and an antiprotease buffer, cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany). One tablet of cOmplete was added to 50 ml of 1X PBS to prepare the extraction solution. A single wound tissue sample was added to 1 ml of the extraction solution and homogenized in 1 ml of the extraction solution using PowerGen Generator (PowerGen 125, Fisher Scientific, Pittsburg, PA) and a saw tooth blades (7X95mm). The homogenate was centrifuged at 1000 rpm for 10 min at 4°C. The supernatant thus obtained was taken up in 3 ml syringes (BD Biosciences, Mexico) and passed through a 1.2 μm Sartorius Minisart Syringe filter (Supelco, Bellfonte, PA). The filtrate (around 600 µl) was aliquoted into tubes and stored at -80°C. A 15µl aliquot was used for protein assay.

Protein Assays

Protein concentrations of tissue extracts were determined by using Bio-Rad Protein Assay –Dye Reagent Concentrate, Bio-Rad Laboratories, Richmond, CA and their protocol was followed. Protein assays were done so that the cytokine concentrations in these samples could be expressed per milligram of protein. Stock standard solution of 1mg/ml was prepared by using bovine albumin purchased from Sigma Chemical Co., St. Louis, MO and dissolved in distilled water. Blank and serial dilutions of standard (1 mg/ml, 0.8 mg/ml, 0.6 mg/ml, 0.4 mg/ml and 0.2
mg/ml) were used to generate the standard curve. The 15 µl aliquot from tissue extraction was
diluted in 30 µl distilled water (total volume 45 µl) and 10 µl of this dilution was used to load a
single well of a Costar 96 well plate. All protein assays were done in triplicate. The dye reagent
concentrate was diluted 1:4 with distilled water and 200 µl of this diluted dye was added to each
of the wells. The plates were incubated on a shaker for 10 min and then absorbance was
measured at 595 nm on a Clariostar plate reader (BMG Labtech, GmbH, Ottenberg, Germany)
and analyzed using Mars Data Analysis Software (Version 3.01R2, 2013 BMG Labtech). A
standard curve was plotted and used for calculating protein concentrations of tissue extracts. The
protein concentrations were expressed as mg/ml.

**Cytokine Analysis**

Bio-Plex Pro Mouse Cytokine 23-plex Assay kit (Bio-Rad Laboratories, Inc.,
Philadelphia, PA) was used for measuring cytokine content using a Bio-Plex 200 instrument (lab
of Dr. Stanislaw Stepkowski, UTMC, Toledo, OH) and Bio-Plex Manager 6.1 software (Bio-Rad
Laboratories, Inc., Philadelphia, PA) was used for machine operation and data collection. A Bio-
Plex handheld magnetic washer (Bio-Rad Laboratories, Inc., Philadelphia, PA) was used for all
washing steps indicated below. The protocol accompanying the kit was followed. First the Bio-
Plex 200 instrument was calibrated and validated. This was followed by reconstituting in 1xPBS
the standard that was provided in powdered form and keeping on ice for 30 min. After
incubation, the standard stock solution was used to make serial four-fold dilutions. Serial
dilutions were done based on the concentrations of the cytokine standards. The Bio-Plex
Manager software generated a different standard curve for each cytokine that then was used for
determining cytokine concentrations in the tissue extracts. All standards and samples were first
placed in a 96 well round bottom plate so that they could be easily transferred on to the assay
plate. The 10x coupled magnetic beads were mixed for 30 secs and then assay buffer was added to dilute it to 1x (for 96 wells, 575 µl of 10x beads were diluted with 5175 µl of assay buffer for a total volume of 5750 µl). The diluted beads were mixed for 10-20 sec and 50 µl was added to each well on the assay plate. An additional 50 µl of wash buffer was added to each well. The plate was washed two times with 100 µl Bio-Plex wash buffer. Following this, 50 µl of standards, sample duplicates and blank quadruplicates was added to each well after mixing. The plate was covered with sealing tape, put in an opaque box to protect from light and incubated at room temperature on a shaker at 850 +/- 50 rpm for 30 min. During incubation, 10x antibodies were prepared by mixing and diluting 1x (for 96 wells, 300 µl of 10x detection Ab was diluted with 2700 µl of detection antibody diluent for a total volume of 3000 µl). After incubation, the plate was washed three times with 100 µl wash buffer, followed by addition of 25 µl of diluted detection Ab to each well. The plate was then covered with sealing tape, put in light protection box and incubated for 30 min at room temperature on a shaker at 850 +/- 50 rpm. Bio-Plex Manager software was installed and values for standards were entered during the incubation period. Also, 100x SA-PE (streptavidin phycoerythrin) was mixed and diluted to 1x (for 96 wells, 60 µl of 100x SA-PE was diluted with 5940 µl of assay buffer for a total volume of 6000 µl). After incubation, the plate was washed with 100 µl wash buffer three times. Fifty µl of diluted SA-PE was then added to each well. Sealing tape was used to cover the plate and the plate was light protected, followed by incubation at room temperature for 10 min on a shaker at 850 +/- 50 rpm. After incubation, the plate was washed with 100 µl wash buffer three times. The beads were then resuspended in 125 µl assay buffer. The plate was sealed and light-protected, followed by incubation at room temperature for 30 secs on a shaker at 850 +/- 50 rpm. The sealing tape was removed and plates were read on the Bio-Plex 200. The following cytokines
were measured: Eotaxin, G-CSF, GM-CSF, IFN-γ: Interleukins (IL’s); IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17A, KC (keratinocyte), MCP-1/MCAF (monocyte chemotactic and activating factor), MIP-1α, MIP-1β (macrophage inflammatory protein), RANTES (regulated on activation, normal T cell expressed and secreted) and TNF-α (tumor necrosis factor alpha).

**Statistics**

Statistical analysis was done using SPSS software (IBM, 2015, version 23). For the cytokine concentration data, general linear model Univariate ANOVA (along with post hoc tests, Bonferroni and Tukey) was performed for PCB, Exercise and Day. Based on the significance of these data, independent T-tests were run between groups within PCB, Exercise and Day. Error bars are +/- 1 standard error of the mean (SEM). Significance was determined as p ≤ 0.05. Note: Before data were selected for running the statistical analysis, in each of the groups the numbers above or below mean +/- 1 standard deviation (SD) were omitted since there were few that were drastically skewing the group means.

**Results**

**Effect of Exercise and PCB (Aroclor 1254) on IL-1β levels**

IL-1β concentrations were determined at Day 3 and Day 5 post-wounding (Fig. 5). Univariate ANOVA revealed following significant interactions: PCB X exercise (F3,51 = 3.956; p = 0.013), PCB X day (F3,51 = 3.707; p = 0.017) and exercise X day (F1,51 = 8.033; p = 0.007). Independent t-tests were performed to further explore the within group interactions.

**Day Effect:** In mice not exercised, administered 100 µg/g PCB, significantly greater concentrations of IL-1β were present post-wounding on Day 5 as compared to Day 3 (#:t6 = -2.640; p = 0.039) (Fig. 5A, B). Mice that were not exercised and administered 1000 µg/g PCB,
exhibited significantly greater concentrations of IL-1β on post-wounding Day 5 as compared to Day 3 ($t_6 = -3.771; p = 0.009$) (Fig. 5A, B). Exercised mice, not administered PCB, revealed significantly less IL-1β on Day 5 as compared to Day 3 ($t_6 = 3.932; p = 0.008$) (Fig. 5C, D).

**Exercise Effect:** Tissue from mice administered 100 µg/g PCB, contained significantly greater concentrations of IL-1β on Day 3 in exercised animals as compared to animals not exercised ($t_6 = -3.309; p = 0.016$) (Fig. 5A, C). Concentrations of IL-1β on Day 5 in mice not administered PCB were significantly less in exercised as compared to not exercised mice ($t_5 = 4.561; p = 0.006$) Exercised mice, not administered PCB, revealed significantly less IL-1β on Day 5 as compared to Day 3 ($t_6 = 3.932; p = 0.008$) (Fig. 5B, D). On Day 5, mice administered 500 µg/g PCB, displayed a trend towards less IL-1β in exercised as compared to not exercised animals ($t_7 = 2.173; p = 0.066$) (Fig. 5B, D). Concentrations of IL-1β on Day 5 in mice administered 1000 µg/g PCB were significantly lower in exercised mice as compared to mice not exercised ($t_5 = 6.722; p = 0.001$) (Fig. 5B, D).

**PCB Effect:** On Day 3 wound tissue from animals not exercised contained significantly less of IL-1β in mice given 100 µg/g PCB as compared to the animals receiving no PCB ($t_6 = 2.982; p = 0.025$) (Fig. 5A). On post-wounding Day 5, not exercised mice revealed significantly greater concentrations of IL-1β in animals administered 1000 µg/g PCB as compared to mice not administered PCB ($t_4 = -10.193; p = 0.001$) (Fig. 5B). Mice that were not exercised, on post-wounding Day 5 revealed significantly greater concentrations of IL-1β in 1000 µg/g PCB administered animals as compared to the ones administered 100 µg/g PCB ($t_5 = -2.898; p = 0.034$) (Fig. 5B). Post-wounding Day 5 mice that were not exercised revealed significantly greater concentrations of IL-1β in 1000 µg/g PCB treated animals as compared to animals administered 500 µg/g PCB ($t_5 = -2.670; p = 0.044$) (Fig. 5B). On post-wounding Day 5,
Fig. 5: Effect of Exercise and PCB (Aroclor 1254) on levels of IL-1β in wound tissue at Day 3 and Day 5 post-wounding: Comparison of IL-1β levels, 3 days post-wounding in animals not exercised reveals a pattern of reduced levels with different doses of PCB as compared to no PCB administration, with PCB 100 demonstrating the most reduction. Similar pattern is observed at Day 3 in exercised animals, but with no change in PCB 100 administration. However, on Day 5, IL-1β levels in not exercised animals reveals a pattern of dose dependent increase. In exercised animals, on Day 5, levels of IL-1β are higher in PCB 100 and 1000 doses, but remain the same with PCB 500 administration.
exercised animals, demonstrated significantly greater concentrations of IL-1β in mice administered 100 µg/g PCB as compared to the animals not administered PCB (¶: $t_6 = -3.678; p = 0.010$) (Fig. 5D). Mice that were exercised, on Day 5 post-wounding revealed significantly greater concentrations of IL-1β in 100 µg/g PCB administered animals as compared to the ones not administered PCB ($\Delta: t_6 = -3.659; p = 0.011$) (Fig. 5D). Post-wounding Day 5 animals that were exercised demonstrated significantly lower concentrations of IL-1β in 500 µg/g PCB administered animals as compared to the ones administered 100 µg/g PCB ($\infty: t_7 = 2.719; p = 0.030$) (Fig. 5D).

**Effect of Exercise and PCB (Aroclor 1254) on IL-6 levels**

IL-6 levels were measured at Day 3 and Day 5 post-wounding (Fig. 6). Univariate ANOVA revealed significant PCB effect ($F_{3,56} = 3.008; p = 0.038$). It also revealed significant interactions of the following: PCB X exercise ($F_{3,56} = 3.113; p = 0.033$), PCB X day ($F_{3,56} = 3.103; p = 0.034$) and exercise X day interaction ($F_{1,56} = 7.400; p = 0.009$). There was a trend towards PCB X exercise X day interaction ($F_{3,56} = 2.408; p = 0.077$). Independent t-tests were performed to further explore the within group interactions.

**Day Effect:** In mice that were not exercised and administered 500 µg/g PCB, there were significantly greater concentrations of IL-6 on post-wounding Day 5 as compared to Day 3 ($\#: t_6 = -5.447; p = 0.001$) (Fig. 6A, B). In mice that were exercised and administered 100 µg/g PCB, there were significantly lower concentrations of IL-6 on Day 5 post-wounding as compared to Day 3 ($*: t_8 = 5.344; p = 0.001$) (Fig. 6C, D).

**Exercise Effect:** Concentrations of IL-6 on Day 3 post-wounding in mice administered 100 µg/g PCB were significantly greater in exercised mice as compared to mice not exercised ($\$: $t_8 = -2.564; p = 0.033$) (Fig. 6A, C). On Day 3 post-wounding, mice administered 1000 µg/g PCB,
revealed significantly lower concentrations of IL-6 in exercised animals as compared to animals not exercised (€:t₈ = 2.458; p = 0.039) (Fig. 6A, C). In mice administered 500 µg/g PCB, on post-wounding Day 5 revealed significantly lower concentrations of IL-6 in exercised animals as compared to animals not exercised (!:t₇ = 5.502; p = 0.001) (Fig. 6B, D).

**PCB Effect:** On post-wounding Day 5, mice not exercised revealed significantly greater concentrations of IL-6 in animals administered 500 µg/g PCB as compared to mice not administered PCB (¥:t₇ = -4.922; p = 0.002) (Fig. 6B). Post-wounding Day 5 animals that were not exercised revealed significantly greater concentrations of IL-6 in 500 µg/g PCB administered animals as compared to the ones administered 100 µg/g PCB (¶:t₇ = -2.862; p = 0.024) (Fig. 6B). Mice that were not exercised, on post-wounding Day 5 demonstrated lower concentrations of IL-6 in 1000 µG/G PCB administered animals as compared to 500 µg/g PCB administered mice (¶:t₇ = 3.073; p = 0.018) (Fig. 6B). Exercised animals, on Day 3 post-wounding, revealed significantly greater concentrations of IL-6 in mice administered 100 µg/g PCB as compared to the animals not administered PCB (€:t₇ = -2.737; p = 0.029) (Fig. 6C). Mice that were exercised, on Day 3 post-wounding demonstrated significantly lower concentrations of IL-6 in 1000 µg/g PCB administered animals as compared to the ones not administered PCB (＆:t₈ = 2.722; p = 0.026) (Fig. 6C). Mice that were exercised, on Day 3 post-wounding revealed significantly lower concentrations of IL-6 in 500 µg/g PCB administered animals as compared to 100 µg/g PCB administered mice (Δ:t₈ = 2.382; p = 0.044) (Fig. 6C). Post-wounding Day 3 mice that were exercised revealed significantly lower concentrations of IL-6 in 1000 µg/g PCB treated animals as compared to animals administered 100 µg/g PCB (∞:t₀ = 6.883; p < 0.001) (Fig. 6C).
Fig. 6: Effect of Exercise and PCB (Aroclor 1254) on levels of IL-6 in wound tissue at Day 3 and Day 5 post-wounding: In not exercised animals, on Day 3, there appears to be no difference in the means of IL-6 levels across PCB doses. However, Day 3, exercised animals demonstrate a pattern of reduced IL-6 levels with PCB 500 and PCB 1000, but increased with PCB 100 administration as compared to PCB 0. On Day 5, in not exercised mice, IL-6 levels appear to be higher with all PCB doses when compared to PCB 0, with PCB 100 being the highest. Whereas, Day 5, exercised animals reveal pattern of slight decrease with all PCB doses as compared to no PCB administration.
**Effect of Exercise and PCB (Aroclor 1254) on KC levels**

KC levels were measured at Day 3 and Day 5 post-wounding (Fig. 7). Univariate ANOVA did not reveal any significant individual or group interactions. Independent t-tests were performed to explore any within group interactions.

**Day Effect:** In mice that were not exercised and administered 500 µg/g PCB, there were significantly greater concentrations of KC on Day 5 post-wounding as compared to Day 3 (*:t_{7} = -2.978; p = 0.021) (Fig. 7A, B).

**Exercise Effect:** On Day 3 post-wounding, mice administered 100 µg/g PCB, revealed trend towards more KC in exercised animals as compared to animals not exercised (t_{7} = -1.956; p = 0.091) (Fig. 7A, C). In mice administered 500 µg/g PCB, on post-wounding Day 3 demonstrated trend towards more KC in exercised animals as compared to animals not exercised (t_{0} = -2.222; p = 0.053) (Fig. 7A, C). Concentrations of KC on Day 3 post-wounding in mice administered 1000 µg/g PCB were significantly lower in exercised mice as compared to mice not exercised (#:t_{6} = 5.011; p = 0.002) (Fig. 7A, C).

**PCB Effect:** On post-wounding Day 3, mice not exercised revealed significantly greater concentrations of KC in animals administered 1000 µg/g PCB as compared to mice administered 100 µg/g PCB (!:t_{6} = -3.014; p = 0.024) (Fig. 7A). Post-wounding Day 3 animals that were not exercised revealed significantly lower concentrations of KC in 1000 µg/g PCB administered animals as compared to the ones administered 500 µg/g PCB (€:t_{7} = -5.122; p = 0.001) (Fig. 7A). Animals not exercised, on Day 5 post-wounding, demonstrated significantly greater concentrations of KC in mice administered 500 µg/g PCB as compared to the animals not administered PCB ($:t_{5} = -2.585; p = 0.049) (Fig. 7B). Mice that were exercised, on Day 3
Fig. 7: Effect of Exercise and PCB (Aroclor 1254) on levels of KC in wound tissue at Day 3 and Day 5 post-wounding: KC levels on Day 3 in not exercised animals reveal pattern of reduction with increasing PCB doses, except PCB 1000 where there is an increase. Similar pattern of reduction in KC levels with increasing dose is seen on Day 3, exercised animals, except for PCB 100 which demonstrates an increase. On Day 5, in not exercised animals, there is a pattern of higher KC with all PCB does as compared to PCB 0, with PCB 500 revealing the highest levels. However, exercised animals on Day 5 demonstrate a pattern of dose dependent reduction in KC levels.
post-wounding revealed significantly lower concentrations of KC in 1000 µg/g PCB administered animals as compared to the ones administered 100 µg/g PCB (£: \( t = 2.520; p = 0.040 \)) (Fig. 7C).

**Effect of Exercise and PCB (Aroclor 1254) on MCP-1 levels**

MCP-1 levels were measured at Day 3 and Day 5 post-wounding (Fig. 8). Univariate ANOVA revealed significant day effect (\( F_{1,53} = 8.623; p = 0.005 \)). It also revealed significant PCB X day interaction (\( F_{3,53} = 3.753; p = 0.016 \)) and significant exercise X day interaction (\( F_{1,53} = 4.247; p = 0.044 \)). Independent t-tests were performed to further explore the within group interactions.

**Day Effect:** In mice that were not exercised and administered 500 µg/g PCB, there was a trend towards more MCP-1 on Day 5 post-wounding as compared to Day 3 (\( t_{8} = -2.299; p = 0.051 \)) (Fig. 8A, B). In mice that were exercised and administered 1000 µg/g PCB, there were significantly lower concentrations of MCP-1 on Day 5 post-wounding as compared to Day 3 (\( *: t_{8} = 3.088; p = 0.015 \)) (Fig. 8C, D).

**Exercise Effect:** Concentrations of MCP-1 on post-wounding Day 3 in mice administered 500 µg/g PCB were significantly greater in exercised as compared to not exercised mice (\( #: t_{7} = -2.460; p = 0.043 \)) (Fig. 8A, C).

**PCB Effect:** Mice not exercised, on Day 3 post-wounding demonstrated significantly greater concentrations of MCP-1 in 1000 µg/g PCB administered animals as compared to the ones administered 500 µg/g PCB (\( !: t_{7} = -2.448; p = 0.044 \)) (Fig. 8A). Animals not exercised, on Day 5 post-wounding, revealed significantly greater concentrations of MCP-1 in mice administered 100 µg/g PCB as compared to the animals not administered PCB (\( \$: t_{6} = -3.320; p = \)
**Fig. 8:** Effect of Exercise and PCB (Aroclor 1254) on levels of MCP-1 in wound tissue at **Day 3 and Day 5 post-wounding:** On Day 3, not exercised animals demonstrate a pattern of increased MCP-1 levels with PCB administration as compared to no PCB administration, except PCB 500 which reveals a decrease. However, on Day 3, in exercised animals, a pattern of reduced KC levels with PCB administration is observed, except PCB 100 where it increases. Day 5, not exercised animals demonstrate a pattern of dose dependent increase in KC levels, except PCB 1000 which is similar to PCB 0. Similarly, exercised animals, on Day 5, have higher KC levels with PCB administration, except PCB 1000 which is lower.
0.016) (Fig. 8B). On post-wounding Day 5, mice not exercised revealed trend towards more MCP-1 in animals administered 500 µg/g PCB as compared to mice not administered PCB ($t_7 = -2.279; p = 0.057$) (Fig. 8B). Day 5 post-wounding animals that were exercised revealed trend towards less MCP-1 in 1000 µG/G PCB administered animals as compared to the ones administered 500 µg/g PCB ($t_5 = 2.532; p = 0.052$) (Fig. 8D).

**Effect of Exercise and PCB (Aroclor 1254) on TNF-α levels**

TNF-α levels were measured at Day 3 and Day 5 post-wounding (Fig. 9). Univariate ANOVA did not reveal any significant individual or group interaction effects. Independent t-tests were performed to further explore the within group interactions.

**Day Effect:** No significant differences in concentrations of TNF-α were revealed between post-wounding Day 5 and Day 3.

**Exercise Effect:** No significant differences in concentrations of TNF-α were observed in animals with or without exercise.

**PCB Effect:** Not exercised animals, on post-wounding Day 3, demonstrated significantly lower concentrations of TNF-α in mice administered 100 µg/g PCB as compared to the animals not administered PCB (*:$t_6 = 2.838; p = 0.030$) (Fig. 9A). On Day 3 post-wounding, exercised mice revealed trend towards less TNF-α in animals administered 100 µg/g PCB as compared to mice not administered PCB ($t_8 = 2.187; p = 0.060$) (Fig. 9C). Mice that were exercised, on Day 3 post-wounding revealed significantly lower concentrations of TNF-α in 500 µg/g PCB administered animals as compared to the ones not administered PCB (#:$t_9 = 2.449; p = 0.037$) (Fig. 9C). Post-wounding Day 3 animals that were exercised revealed trend towards less TNF-α in 1000 µg/g PCB administered animals as compared to the ones not administered PCB ($t_9 = 2.169; p = 0.058$) (Fig. 9C).
Fig. 9: Effect of Exercise and PCB (Aroclor 1254) on levels of TNF-α in wound tissue at Day 3 and Day 5 post-wounding: On Day 3, not exercised animals demonstrate reduced TNF-α levels with varying doses of PCB administration as compared to no PCB. Similarly, reduced TNF-α levels were seen with PCB administration in exercised animals on Day 3. On Day 5, in not exercised animals, PCB 100 revealed a slight increase in TNF-α levels, whereas PCB 500 and PCB 1000 demonstrated little or no change when compared to no PCB. However, on Day 5, exercised mice, reveal little or no change with varying doses of PCB.
Discussion

It has been previously demonstrated that suppression of pro-inflammatory cytokines in the wound tissue can accelerate wound healing (Szpaderska et al., 2003, Keylock et al., 2008). In the present study, comparison of means of the concentrations of the pro-inflammatory cytokines IL-6, KC and MCP-1 in animals not exercised, reveals a pattern of depression at post-wounding Day 3 followed by an increase at Day 5 with 100 µg/g PCB and 500 µg/g PCB administration. On the other hand, this pattern is reversed in 1000 µg/g PCB administered animals, where the amounts of tissue IL-6, KC and MCP-1 are greater at Day 3 and less at Day 5. Means of IL-1β and TNF-α concentrations did not follow similar patterns. It may be that the depression of pro-inflammatory cytokines on Day 5 in PCB administered animals that were not exercised is the reason that wounds healed more rapidly in animals administered 1000 µg/g PCB as compared to the other PCB doses (Fig. 3C, E and F). In a previous study PCB have been shown to compromise macrophage cell viability and increase rates of apoptosis (Ferrante, et al., 2011). A study in knockout mice, a complete absence of macrophages and neutrophils, wound healing occurs more rapidly without scarring (Martin and Leibovich, 2005). This effect of PCB on macrophages is likely more obvious at elevated doses, resulting in more rapid wound healing in animals not exercised and given 1000 µg/g PCB in the present study. In exercised animals, the concentrations of IL-6, KC and MCP-1 were depressed with 500 µg/g PCB and 1000 µg/g PCB, but elevated with 100 µg/g PCB as compared to animals not administered PCB. A pattern of reduction in the content of these cytokines is also seen on Day5, except MCP-1 amount on Day 5 is greater in 100 µg/g PCB and 500 µg/g PCB treated animals. Elevation of the tissue content of the pro-inflammatory cytokines on Day 3 by 100 µg/g PCB could be retarding wound healing in these animals as compared to the other doses of PCB (Fig. 4A, D, E). Also, Day 5 post-
wounding in mice not exercised and Day 3 post-wounding in exercised mice demonstrated a similar pattern of IL-6, KC and MCP-1 cytokine content, which could be the result of the inflammation phase occurring at an earlier stage in exercised mice, and that is contributing to the pattern of more rapid wound healing observed in exercised animals administered 0 µg/g PCB, 500 µg/g PCB and 1000 µg/g PCB (Fig. 2A, C, D). Elevated content of these pro-inflammatory cytokines on Day 3 in exercised mice administered 100 µg/g PCB, may be the reason for depressed wound healing rates observed initially as compared to the mice that were not exercised (Fig. 2B). Keylock et al. (2007) have found that aged mice that were exercised heal more rapidly post-wounding as compared to sedentary mice. However, in younger mice like those used in the present study exercise did not significantly alter wound healing rate, but produced a pattern of more rapid wound healing in the rates. Similarly, the present study revealed patterns of more rapid wound healing but no significant differences. Previous studies have also suggested that reducing the strength of response and length of the inflammatory phase or hastening the resolution phase of wound healing could lead to more rapid wound healing (Martin and Leibovich, 2005; Serhan et al., 2007).
CHAPTER III: EFFECT OF PCB AND EXERCISE ON AVERAGE BODY TEMPERATURE FOLLOWING LPS STIMULATION

Introduction

PCB and exercise effect on the systemic immune response was studied by lipopolysaccharide (LPS) stimulation. LPS is part of the outer membrane of gram negative bacteria. (Patel et al., 2017). LPS plays a protective role in bacteria by acting as its defense system and protecting it from environmental stressors. Thus, the very survival of the organism is dependent on this system (Silipo et al., 2010). The presence of phosphate groups in the anchoring domain (Lipid A) provides rigidity to the outer membrane (Alexander, et al., 2001). The membrane consists of an anchoring domain called Lipid A, a core region which is an oligosaccharide and a polysaccharide region called as the O region. The lipid A domain is also called endotoxin and responsible for many of the immune system responses of LPS. The O region is highly variable among bacteria and is responsible for the evasive mechanisms they employ (Raetz and Whitfield, 2002). Smooth (S) form LPS contains a long O region whereas rough (R) form LPS contains a truncated or absent O region. These forms have different effects on the immune system. Innate immune response plays a major role in LPS identification and its response. Since the LPS is highly conserved, the major LPS binding is done by toll like receptor -4 (TLR-4) which is highly conserved and initiates a prompt response to eliminate the bacteria (Steimle, et al., 2016). This must be done in a timely manner to prevent the harmful effects on the host (Janeway, et al., 2002; Beutler and Rietschel, 2003). If uncontrolled it can even lead to septic shock (Voss et al., 2016). Lipid A structure differs among bacteria and this determines the nature of the specific immune response elicited. Immune responses are based on the pro-inflammatory effects and are classified as agonistic, weak agonistic or antagonistic. The number
of acyl chains on the Lipid A determines the strength of the response, the ones with six acyl chains showing strong agonistic response. *Escherichia coli* (*E. coli*) LPS has 6 acyl chains and elicits one of the strongest immune responses (Steimle, *et al.*, 2016; Rietschel *et al.*, 1993).

Previous *in vitro* and *in vivo* studies have demonstrated that the endotoxic effects of LPS are the result of stimulation of macrophages leading to the production of proinflammatory cytokines like IL-1, IL-6, TNF-α and increase in nitric oxide production (NO) (Hirohashi and Morrison, 1996; Netea *et al.*, 2002; Rodewohl *et al.*, 2017). All of these changes lead to fever by acting on the preoptic-anterior hypothalamic area (POA) which is a regulator of body temperature (Blatteis, 2006). Hence, LPS has been used to study systemic inflammation, by using an infrared camera to measure body temperature changes.

PCB have been shown to suppress the immune response to LPS stimulation by reducing the effect of LPS on macrophages by decreasing TLR-4 receptor mRNA as well as reduction in the content of IL-6, TNF-α, MCP-1 and nitric oxide (Hong *et al.*, 2004; Yoshitake *et al.*, 2008; Santoro, *et al.*, 2015). Exercise reduces the levels of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α following LPS administration (Nogueira *et al.*, 2017). All these interactions of PCB, exercise and LPS stimulation allow examination of the effects of PCB and exercise on the systemic immune response.

**Materials and Methods**

Animal care, PCB exposure, and exercise regimen were identical to that mentioned in Chapter I.

**Euthanasia**

The animals were euthanized by rapid CO2 asphyxiation on Day 8 post-PCB administration, following which their organ weights including the spleen and the thymus were
determined. The animals were decapitated and their blood collected for further analysis of immune response markers.

**Lipopolysaccharide Administration**

LPS acts as an antigen and causes a systemic immunological response. Animals (Table 10) were administered an intraperitoneal injection of lipopolysaccharide from *E. coli* (LPS, 50 µg in 200 µl total volume of phosphate buffered saline; Sigma, St. Louis, MO) 2 days after PCB exposure.

**Average Body Temperature Measurement**

RAZR-IR Nano camera from Sierra Pacific Innovations (Las Vegas, NV) was used to take pictures of the mice for later analysis using the accompanying software to measure the average body temperatures. RAZR-IR infrared camera analyzer software was used to develop an outline around the infrared image of the mouse and to give the average temperature inside the outlined area.

**Statistics**

Temperature on Day 0 (day of PCB administration) over all the groups was normalized to 0 and all the values for the following days were temperature changes as compared to Day 0. Non parametric tests were run on the data. For the PCB and exercise effect, independent samples Kruskal-Wallis test were run. For the day effect, related samples Friedman’s two way analysis of variance by ranks were run.
Table 3: Detailed distribution of the mice for LPS stimulation study

<table>
<thead>
<tr>
<th>LPS Stimulation</th>
<th>PCB doses (µg/g) + LPS</th>
<th>No Exercise</th>
<th>Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 + LPS</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>100 + LPS</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>500 + LPS</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>1000 + LPS</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>
Results

In general, Friedman’s ANOVA did not yield any significant results (except in Fig. 10). After plotting of graphs, it was observed that on Day 6, there were major temperature changes that were not being detected on the non-parametric tests. Univariate ANOVA was run for Day 6, followed by independent t-tests to identify within group interactions. Univariate ANOVA on Day 6, showed significant exercise effect (F1,39 = 4.568; p = 0.039) and PCB X exercise interaction (F3,39 = 4.876; p = 0.006) and trend for PCB effect (F3,39 = 25.42; p = 0.070). No other days showed significant differences or trends in average body temperatures.

Body Temperature Changes after PCB and LPS administration

The average body temperatures from all the animals (Fig. 10) irrespective of the PCB doses or their exercise status were compared (Friedman’s Two-Way ANOVA, F10 = 68.6; p < 0.001). One day after administration of PCB (Day 1) there was a significant decrease in average body temperature (\( W = 2.872; p = 0.001 \)) similar to that observed 3 hours after LPS administration (Day 2 – 3hr) (\( ^\& : W = 3.330; p < 0.001 \)) compared to Day 0. A similar trend of decrease in average body temperatures was observed on Day 4 (\( W = 2.234; p = 0.060 \)) as compared to Day 0. Day 3 and Day 5 show significant increase in average body temperature as compared to Day 1 (\( ^1 : W = -3.085; p < 0.001 \) & \( ^1 : W = -3.160; p < 0.001 \) respectively), Day 2 - 3hr (\( ^\& : W = -3.543; p < 0.001 \) & \( ^\& : W = -3.617; p < 0.001 \) respectively), and Day 4 (\( ^\#: W = 2.447; p = 0.019 \) & \( ^\#: W = -2.521; p = 0.013 \) respectively). However on Day 8 the average body temperatures are significantly lower than Day 1 (\( ^1 : W = -2.734; p = 0.004 \)) and Day 2 - 3hr (\( ^\& : W = -3.191; p < 0.001 \)).
Fig 10: Comparison of Changes in Average Body Temperature Post LPS Administration in Animals from Day 0 (Day of PCB Administration) to Day 8 (n = 47-48): The data shows temperature changes after PCB and LPS administration in all the animals in this part of the study. The body temperature decreases immediately after both PCB and LPS administration. However, temperature gradually increases after 6 hours of LPS administration and is highest on Day 3 and Day 5, after which it gradually decreases.
Effect of Exercise on Body Temperature Changes with no PCB Treatment followed by LPS administration

Independent t-tests yielded the following results: In animals that were not administered PCB (Fig. 11 A), after LPS administration, the change in average body temperature on Day 6 (4 days after LPS administration) in the exercised mice is significantly lower as compared to animals not exercised (**: t₀ = 3.325; p = 0.009).

Effect of Exercise on Body Temperature Changes with Treatment with Varying Doses of PCB followed by LPS administration

Fig. 11B (animals administered PCB 100), Fig. 11C (animals administered PCB 500) and Fig. 11D (animals administered PCB 1000): No significant differences or trends in average body temperatures on days after LPS administration were observed in between exercise and no exercise group of animals.

Changes in Average Body Temperatures with varying doses of PCB followed by LPS administration in Not Exercised Animals

Independent t-tests yielded the following results: The average body temperature significantly higher on Day 6 (4 days after LPS administration) in animals not administered PCB as compared to PCB 100 (**: t₀ = 2.864; p = 0.019), PCB 500 (**: t₀ = 2.964; p = 0.016) and PCB 1000 (**: t₀ = 2.485; p = 0.035) administered mice (Fig. 12A, B, C). No significant differences or trends and no discernible difference in average body temperatures were observed when the other doses of PCB were compared (Fig. 12D, E, F).
Fig. 11: Effect of Exercise on Changes in Average Body Temperatures after LPS administration in mice (n = 5-6): Only not exercised mice that were not administered PCB show a significant increase in body temperature after LPS administration on Day 6 as compared to the exercised mice. In all other PCB treatment groups there is no exercise vs no exercise effect.
Fig. 12: Comparison of Changes in Average Body Temperatures after LPS Administration in Animals Not Exercised and Administered Varying Doses of PCB (n = 5-6): In the not exercised group, on Day 6, average body temperatures are significantly higher in animals administered no PCB as compared to all the other doses of PCB.
Fig. 13: Comparison of Changes in Average Body Temperatures after LPS Administration in Exercised Animals Administered Varying Doses of PCB (n = 5-6): There were no significant changes in body temperature observed between the different PCB doses following LPS stimulation in exercised mice.
Changes in Average Body Temperatures with varying doses of PCB followed LPS administration in Not Exercised Animals

Independent t-tests yielded the following results: The average body temperature appears to be higher on Day 6 (4 days after LPS administration) in PCB 100 treated animals as compared to mice not administered PCB, though no significant differences or trends were observed (Fig. 13A). No significant differences or trends and no discernible difference in means were observed when the other doses of PCB were compared (Fig. 13B, C, D, E, F).

Discussion

Previous studies have found a decrease in core body temperature following PCB administration (Seo and Meserve, 1995; Komives and Alayoku, 1980) which was also observed in the present study as well. The temperature decrease was attributed to reduction in the levels of circulating tetraiodothyronine (T4) levels and reduced oxygen consumption (Seo and Meserve, 1995). On the other hand LPS has been shown to have pyrogenic effects and does that mainly through activating macrophages and increasing the production of various pro-inflammatory cytokines (Blatteis, 2006). However, previous studies have shown that PCB impairs immune response to LPS by reducing the production of nitric oxide (NO) and pro-inflammatory cytokines IL-6, TNF-α, MCP-1 (Hong et al., 2004; Yoshitake et al., 2008; Santoro, et al., 2015). Similarly, exercise has been demonstrated to reduce the generation of pro-inflammatory cytokines like IL-1β, IL-6 and TNF-α after LPS administration (Nogueira et al., 2017). However, in LPS stimulated animals exercise also increased the content of hydrogen sulfide (H₂S) levels in the POA which is important for thermoregulation. In the same study, the rats were exercised on a treadmill for 4 weeks and their deep body temperatures were measured for 5 hours and cytokines were measured 2 hours after LPS stimulation. Despite these changes in cytokines and H₂S
concentrations, no significant difference in the body temperature was observed in the sedentary and exercised animals (Nogueira et al., 2017). Similarly, in the present study, no significant differences between exercise and no exercise were observed after LPS administration in animals given no PCB administration in the initial days (Fig. 11). However, exercised animals had significantly lower average body temperature as compared to the animals not exercised, 4 days after LPS administration (Day 6). This could be because the animals body temperatures were measured for a longer period of time in the present study. In animals administered PCB doses 100, 500 and 1000 µg/g, exercise caused no change after LPS administration. The present study shows that in animals not exercised that were LPS stimulated, only those animals administered no PCB had significantly higher body temperatures at Day 6 as compared to PCB 100, PCB 500 and PCB 1000 treated animals (Fig. 12), which is similar to studies where diminished LPS response was observed as a result of PCB administration. However, when the animals were exercised (Fig 13), LPS stimulation did not significantly alter body temperatures irrespective of the doses of PCB. This finding could be a result of combined effect of PCB and exercise to reduce the pro inflammatory cytokines and other factors responsible for LPS response.
CONCLUSION

Two models namely the threshold model and linear threshold model (LNT) have been used to assess risk in toxicology for non-carcinogens and carcinogens respectively. Biphasic model has been proposed as the third alternative and appears to be a better fit for the dose-response effect (Calabrese and Baldwin, 2003; Hashmi et al., 2014). This biphasic model has been called hormesis, where the lower dose shows stimulation whereas the higher dose shows inhibition. PCB have been shown to demonstrate hormesis in mainly non-coplanar PCB.

Oxidative stress by production of reactive oxygen species further causing apoptosis is one of the major factors implicated for the hormentic response of PCB (Hashmi et al., 2015). In the present study we used, we did not observe any hormetic effects. Aroclor 1254 which is a mixture of PCB congeners containing both coplanar and non-coplanar PCB and this could be one of the reasons why we did not see the hormetic effects.

Corn oil was used as a vehicle for PCB in the present study and it was store bought. Several studies have used corn oil for i.p. injections like the present study. Some studies mention the source of the corn oil from a chemical company (Lai et al., 2010; Shen et al., 2014; Bunaciu et al., 2007), whereas some don’t mention the source (Shen et al., 2016; Selvakumar et al., 2013), including the Harper et al., 1995 paper that was used as a reference for the present study. All animals in the present study received corn oil from the same bottle as vehicle and thus any impurity or contamination should produce similar changes in the treatment groups and should not affect the differences between the treatment groups.

The wounds created in mice in the present study were done under sterile conditions. After the procedure the mice were transferred to cages with clean bedding and their cages and bedding were regularly changed. However, no further aseptic precautions were taken in terms of bedding
or cages. They were also not placed in a room that had special sterile circulation. Keylock et al., 2008 studied wound healing and housed the mice in a similar setting as the present study. We did not observe any signs of infection in the mice. Since all mice were exposed to similar environment, we believe treatment group differences should not be affected.

Wound healing has been studied in relation to sex hormones. Effect of testosterone was studied in castrated mice and it was observed that the wounds in these animals healed faster as compared to the sham castrated animals. Similar delayed wound healing was observed in human study with increasing testosterone levels (Ashcroft and Mills, 2002). Androgens have been shown to have suppressive effect on B-cells and macrophages reducing their ability to produce IL-1 and IL-6 (Olsen and Kovacs, 1996; Angele et al., 1998). Androgens have been shown to act on smad3, which acts as a mediator for TGF-β and increases inflammation and slows epithelization thus slowing wound healing (Ashcroft et al., 2003). Effect of estrogen was studied in ovariectomized rats and it was observed that wound healing was slower in these animals as compared to the control group (Ashcroft et al., 1997). Wound healing study in elderly humans has shown that topical estrogen improves wound healing rates (Ashcroft et al., 1999). Estrogen has been shown to have an effect through the regulation of macrophage migration inhibitory factor (MIF) which is produced by monocytes, keratinocytes and endothelial cells (Ashcroft et al., 2003; Abe et al., 2000). In this study, we have used female mice and neither their estrous cycle nor the levels of estrogen were measured during wound healing.

Wound healing has been studied in different age groups, especially the older population. Studies have shown that wound healing is hampered in aged population as result of changes occurring in all the stages of wound healing (Sgonc and Gruber, 2013; Ashcroft et al., 2002). Keylock et al., 2008 has shown that wound healing was faster in older mice that were exercised
as compared to mice that were not exercised. However, in younger mice exercise did not show significant change in wound healing when compared to the not exercised animals. In the present study, we used young mice (8 weeks old) and that could be one of the reasons that we did not observe significant changes in wound healing with exercise.

The overall goal of this study was to evaluate the effect of PCB and exercise on the immune system, both local and systemic. This interrelationship has not been previously studied. Wound healing was used to examine the local immune response. Our wound healing data show that only the greatest dose of PCB, 1000 µg/g produces a pattern of more rapid wound healing in animals not exercised. In exercised animals, the least amount of PCB, 100 µg/g has the slowest wound healing rates. Exercised animals generated more rapid wound healing with no PCB, 500 µg/g and 1000 µg/g of PCB and slower rates with 100 µg/g PCB administration. This shows that at greatest amount, 1000 µg/g PCB has a similar effect on reducing wound size to exercise, whereas the least amount, 100 µg/g PCB elicits a compensatory mechanism and slows wound healing rates in exercised mice. This study could not compare the time required by wounds to completely heal (10% of original wound size), since wounds scabbed in many mice and sample size was insufficient to ascribe significance. More animals will be required to validate the patterns mentioned above in the wound size measurement data. Also, the robust and adaptive immune system in these young mice could be a reason that the results are not as clear as observed in older mice in previous studies.

The cytokine concentrations obtained at Day 3 and Day 5 post-wounding provided a better understanding of the changes occurring at the wound tissue. Most of the changes observed in the wound sizes could be correlated with the changes in the pro-inflammatory cytokines (mainly IL-6, KC and MCP-1) occurring in the wound tissue. In exercised animals the pattern of
cytokine content on Day 3 were similar to Day 5 in animals not exercised, which may be a result of the inflammatory phase occurring earlier in exercised animals and also the reason for the pattern of more rapid wound healing. Tissue from animals not exercised, administered 1000 µg/g PCB contain elevated concentrations of pro-inflammatory cytokines on Day 3 post-wounding, but much lower on Day 5, very similar to that observed in exercised animals, and could be a reason for most rapid wound healing pattern. Tissue from exercised animals administered 100 µg/g contained the most of pro-inflammatory cytokines on Day 3, which could the reason for them having the pattern for slowest wound healing. The sample size has been a restrictive factor in obtaining significant results. However, the present study illustrates that cytokine concentrations in wound tissue could be used as a productive tool for analyzing wound healing rates.

The systemic immune response was studied using LPS stimulation and measuring average body temperatures. The measurement of average body temperature using an infrared camera, is a novel approach as compared to studies that measure core body temperature. Maximum temperature was located in different areas of the body on different days, but did not follow any set pattern among the groups and this would seem to provide an advantage with measuring average body temperatures rather than measuring temperature at one particular point. Another novel part of this study is that average body temperatures were measured for 6 days post LPS injection as compared to studies measuring temperatures for only a few hours. In mice not exercised with no PCB administration, LPS caused a gradual increase in temperature, peaking 4 days after LPS injection and then gradually falling back to pre LPS levels. This pattern is not observed in mice not exercised administered different doses of PCB, only temperature fluctuations are seen, but never a consistent rise or fall. This can be explained by studies that
demonstrate that PCB inhibits macrophage viability and function which are essential for the immune response to LPS stimulation. Exercise shows a pattern of dampening the temperature rise seen in animals not administered PCB, but has little effect on the temperature measurements after administration of different doses of PCB. Macrophages play a central and crucial role in innate immunity. Impairment of macrophage function and the subsequent depression of the immune response can make animals including humans more susceptible to infections.

Macrophages play a very important role in the immune system and demonstrate plasticity, which means that they have phenotypes into which they can transform depending on the stimuli. LPS or IFN-γ can shift the macrophages into M1 phenotype, whereas IL-4, IL-10 and IL-13 can shift them into M2 phenotype (Mills et al., 2000; Martinez et al., 2008). In wound healing, they are initially in the M1 phenotype which is more pro-inflammatory releasing IL-1β, IL-6, IL-12 and TNF-α. In the later stages, they are in the M2 phenotype which has more anti-inflammatory effect releasing IL-4, IL-10, IL-13 and TGF-β (Noël et al., 2004; Benoit et al., 2008; Mantovani et al., 2007).

Overall, it appears that both PCB and exercise may exert their effects on the macrophages and thereby altering the level of pro-inflammatory cytokines secreted by them. Studies have shown that PCB decreases the viability of macrophages and also increases their apoptosis (Ferrante et al., 2011). Whereas, exercise reduces the number of toll like receptors on the macrophages and aids in conversion of macrophages from M1 to M2 phase and thus reduce secretion of pro-inflammatory cytokines and increase anti-inflammatory cytokines (Gleeson et al., 2011). The decreased survival of macrophages (which plays a central role in recruiting other immune cells) as seen with PCB exposure will expose the organism to increased chances of infection. For example, studies in Dutch preschool children have shown that PCB exposure can
lead to increased middle ear infections (Weisglas-Kuperus et al. 2000; Weisglas-Kuperus et al., 2004). However, moderate intensity exercise has been shown to reduce the chances of upper respiratory tract infections. It will be interesting to see how animals with wounds that are infected or have systemic infection will respond to PCB and exercise.

PCB exposure has been associated with insulin resistance and thus increased risk for diabetes (Lee et al., 2007; Silverstone et al., 2012). Diabetes hampers the healing of wounds leading to chronic wounds which are a serious health concern in these patients and reduces quality of life and treatment causes a huge financial burden (Tsourdi et al., 2013; Margolis et al., 2005). Studies have demonstrated that exercise improves glycemic control and insulin sensitivity in diabetes patients (Ishii et al., 1998; Boulé et al., 2001). This along with the finding that exercise accelerates wound healing (Keylock et al., 2008), could make exercise an invaluable tool to counter the adverse effects of PCB and needs to be further investigated.
REFERENCES


APPENDIX A: EFFECT OF PCB AND EXERCISE ON ADDITIONAL CYTOKINES FROM WOUND TISSUE

The figures contain comparisons of cytokines for which there were either no statistically different values regardless of exercise or PCB administration or their role in the wound healing process in not clear.

Fig. A-1: Effect of Exercise and PCB (Aroclor 1254) on levels of Eotaxin in wound tissue at Day 3 and Day 5 post wounding: Univariate ANOVA showed significant PCB main effect and significant exercise X day interaction. Independent t-tests revealed the following within group interactions. Significance was determined as $p \leq 0.05$ and trend (not indicated in the figure) was determined as $p = 0.051$ to 0.1 ($n = 3$-$6$ mice). Day Effect: No significant differences or trends in
Eotaxin levels between Day 5 and Day 3. **Exercise Effect:** No significant differences or trends in Eotaxin levels between animals with or without exercise. **PCB Effect:** Significant: In exercised mice, at Day 3, Eotaxin levels in PCB 100 < PCB 0 (*: t_6 = 3.425; p = 0.014) and PCB 500 < PCB 0 ( #: administered animals. In exercised mice, at Day 5, Eotaxin levels in PCB 500 < PCB 0 ($) administered animals.
**Day 3 and Day 5 post wounding:** Univariate ANOVA showed significant PCB X exercise interaction. Independent t-tests revealed the following within group interactions. Significance was determined as $p \leq 0.05$ and trend (not indicated in the figure) was determined as $0.051 < p < 0.1$ ($n = 3$-6 mice). **Day Effect:**

a) **Significant:** In not exercised administered PCB 500, G-CSF levels on Day 5 > Day 3(*). In exercised mice administered PCB 1000, G-CSF levels on Day 5 < Day 3(#).

b) **Trends:** In mice, not exercised and not administered PCB, G-CSF levels on Day 5 < Day 3. In mice that were exercised and not administered PCB, G-CSF levels on Day 5 > Day 3. In exercised mice, administered PCB 100, G-CSF levels on Day 5 > Day 3.

**Exercise Effect:**

a) **Significant:** On Day 5 in mice not administered PCB, G-CSF levels in exercised > not
exercised animals (>). In mice administered PCB 100, on Day 5, G-CSF levels in exercised > not exercised animals (!). b) Trends: On Day 5, mice administered PCB 500, G-CSF levels in exercised < not exercised animals. G-CSF levels in exercised < not exercised mice, on Day 5 in animals administered PCB 1000. PCB Effect: a) Significant: In Day 3, exercised mice levels of G-CSF in animals administered PCB 100 > PCB 0 (£). In Day 5 animals, not exercised, G-CSF levels in PCB 500 > PCB 0 administered animals (£). In exercised mice on Day 5, G-CSF levels in PCB 1000 < PCB 0(¶). In not exercised mice on Day 3, G-CSF levels in PCB 500 < PCB 100 administered animals (£). Exercised mice on Day 3 revealed levels of G-CSF in PCB 1000 < PCB 100 administered animals (©). In exercised mice on Day 5, G-CSF levels in PCB 1000 < PCB 100 administered animals (¶). b) Trends: In mice not exercised, on Day 3, G-CSF levels in PCB 500 < PCB 0 administered animals. In exercised animals on Day 5, levels of G-CSF in PCB 500 < PCB 100 administered mice. On Day 5, in exercised mice G-CSF levels in PCB 1000 < PCB 500 administered animals.
Fig. A-3: Effect of Exercise and PCB (Aroclor 1254) on levels of GM-CSF in wound tissue

at Day 3 and Day 5 post wounding: Univariate ANOVA showed significant PCB effect, trend for exercise effect, significant exercise X day interaction and significant PCB X exercise X day interaction. Independent t-tests revealed the following within group interactions. Significance was determined as $p \leq 0.05$ and trend (not indicated in the figure) was determined as $0.051 \leq p < 0.1$ ($n = 3-6$ mice). **Day Effect: Trends:** In mice, not exercised and not administered PCB, GM-CSF levels on Day 5 > Day 3. In exercised mice, not administered PCB, GM-CSF levels on Day 5 < Day 3 ($t_{5} = 2.516; p = 0.053$). **Exercise Effect: Significant:** GM-CSF levels on Day 3 in mice not administered PCB in exercised > not exercised mice (*). In mice administered PCB 100, on
Day 3, GM-CSF levels in exercised > not exercised animals (#). **PCB Effect:** a) Significant: On Day 3, in exercised mice, GM-CSF levels in PCB 100 < PCB 0 administered animals ($). Mice not exercised, on Day 3, levels of GM-CSF in PCB 500 < PCB 0 administered animals(†). Day 5 animals, not exercised, GM-CSF levels in PCB 500 < PCB 0 administered mice (£). In exercised mice, on Day 3, GM-CSF levels in PCB 500 < PCB 0 administered animals (€). In Day 3 mice, not exercised, GM-CSF levels in PCB 1000 < PCB 0 administered mice (¥). In mice that were not exercised, on Day 5 levels of GM-CSF in PCB 1000 < PCB 0 administered animals (®). In exercised mice on Day 3, GM-CSF levels in PCB 1000 < PCB 0 administered animals (¶). In exercised mice on Day 5, levels of GM-CSF in PCB 1000 < PCB 0 administered animals (∆). In exercised animals on Day 5, GM-CSF levels in PCB 500 < PCB 100 administered mice (∞). b) Trends: In not exercised animals, on Day 5, GM-CSF levels in mice administered PCB 100 < PCB 0. In not exercised mice on Day 3, levels of GM-CSF in PCB 1000 < PCB 100 administered animals. In exercised mice on Day 3, levels of GM-CSF in PCB 1000 < PCB 100 administered animals (t_{8} = 2.289; p = 0.051).
**Fig. A-4: Effect of Exercise and PCB (Aroclor 1254) on levels of IFN-γ in wound tissue at Day 3 and Day 5 post wounding:** Univariate ANOVA showed trend towards PCB X exercise X day interaction. Independent t-tests revealed the following within group interactions.

Significance was determined as $p \leq 0.05$ and trend (not indicated in the figure) was determined as $p = 0.051$ to 0.1 ($n = 3$-6 mice). **Day Effect:** 

**a)** Significant: Mice not exercised and administered PCB 500, levels of IFN-γ on Day 5 < Day 3(*). In exercised mice administered PCB 1000, IFN-γ levels on Day 5 < Day 3(>).

**b)** Trends: In not exercised mice, administered PCB 1000, levels of IFN-γ on Day 5 > Day 3. **Exercise Effect:** 

**a)** Significant: Levels of IFN-γ in exercised < not exercised mice, on Day 3 animals administered PCB 1000($).

**b)** Trends: On
Day 5, mice administered PCB 500, levels of IFN-γ in exercised > not exercised animals. **PCB Effect:** 

*a) Significant:* In mice not exercised, on Day 3, levels of IFN-γ in PCB 500 < PCB 0 administered animals (!). In exercised mice, on Day 3 IFN-γ levels in PCB 1000 > PCB 100 administered animals (£). On Day 3, in animals that were not exercised, levels of IFN-γ in PCB 1000 < PCB 500 administered mice (€). In exercised mice on Day 3, IFN-γ levels in PCB 1000 > PCB 500 administered animals (¥). 

*b) Trends:* On Day 3, in animals not exercised levels of IFN-γ in PCB 500 > PCB 100 administered animals. In mice not exercised, on Day 5, levels of IFN-γ in PCB 1000 > PCB 500 administered animals.
Fig. A-5: Effect of Exercise and PCB (Aroclor 1254) on levels of IL-1α in wound tissue at Day 3 and Day 5 post wounding: Univariate ANOVA showed significant PCB effect, trend for exercise effect, significant day effect, and significant PCB X exercise X day interaction. Independent t-tests revealed the following within group interactions. Significance was determined as $p \leq 0.05$ and trend (not indicated in the figure) was determined as $p = 0.051$ to 0.1 ($n = 3$-$6$ mice). **Day Effect: Significant:** In mice that were not exercised and not administered PCB, levels of IL-1α on Day 5 > Day 3($^*$). In exercised mice, administered PCB 500, IL-1α levels on Day 5 > Day 3($^#$). **Exercise Effect: Significant:** On Day 5 in mice not administered PCB levels of IL-1α in exercised < not exercised mice ($^\$$). On Day 5, mice administered PCB
levels of IL-1α in exercised > not exercised animals (!). **PCB Effect:** Significant: On Day 5, in animals not exercised, levels of IL-1α in PCB 500 < PCB 0 administered mice (£). In mice not exercised, on Day 5, levels of IL-1α in PCB 1000 < PCB 0 administered animals (€).
Fig. A-6: Effect of Exercise and PCB (Aroclor 1254) on levels of IL-2 in wound tissue at Day 3 and Day 5 post wounding: Effect of Exercise and PCB (Aroclor 1254) on IL-2 levels:

Univariate ANOVA showed significant exercise effect. Independent t-tests revealed the following within group interactions. Significance was determined as $p \leq 0.05$ and trend (not indicated in the figure) was determined as $p = 0.051$ to 0.1 ($n = 3-6$ mice). **Day Effect:**

*Significant*: In mice not exercised and administered PCB 500, levels of IL-2 on Day 5 < Day 3(*). **Exercise Effect**: *Significant*: On Day 5, in mice administered PCB 500, levels of IL-2 in exercised > not exercised mice (#). **PCB Effect**: No significant differences or trends in IL-2 levels between different doses of PCB.
Fig. A-7: Effect of Exercise and PCB (Aroclor 1254) on levels of IL-3 in wound tissue at Day 3 and Day 5 post wounding: Univariante ANOVA showed significant PCB effect.

Independent t-tests revealed the following within group interactions. Significance was determined as \( p \leq 0.05 \) and trend (not indicated in the figure) was determined as \( p = 0.051 \) to 0.1 (\( n = 3-6 \) mice). **Day Effect:** No significant differences or trends in IL-3 levels between Day 5 and Day 3. **Exercise Effect:** Significant: On Day 5, in mice administered PCB 500 levels of IL-3 in exercised < not exercised mice (*). **PCB Effect:** a) Significant: In not exercised animals, on Day 5, levels of IL-3 in PCB 100 < PCB 0 administered mice (#). Mice exercised, on Day 3, IL-3 levels in PCB 500 < PCB 0 administered animals ($). In mice that were exercised, on Day 3,
levels of IL-3 in PCB 500 < PCB 100 administered animals. On Day 3, in mice that were not exercised, IL-3 levels in PCB 1000 > PCB 100 administered animals. b) Trends: On Day 3, in exercised mice, IL-3 levels in PCB 100 < PCB 0 administered animals. In Day 3 animals that were exercised, levels of IL-3 in PCB 1000 < PCB 0 administered mice. In mice that were not exercised, on Day 3, IL-3 levels in PCB 1000 > PCB 500 administered animals.
Fig. A-8: Effect of Exercise and PCB (Aroclor 1254) on levels of IL-4 in wound tissue at Day 3 and Day 5 post wounding: Univariate ANOVA showed significant PCB effect, significant day effect and significant exercise X day interaction. Independent t-tests revealed the following within group interactions. Significance was determined as p ≤ 0.05 and trend (not indicated in the figure) was determined as p = 0.051 to 0.1 (n = 3-6 mice). Day Effect: Trends: In mice that were not exercised and administered PCB 100, levels of IL-4 on Day 5 > Day 3. In mice that were exercised and administered PCB 100, IL-4 levels on Day 5 < Day 3. In mice that were not exercised and administered PCB 1000, levels of IL-4 on Day 5 < Day 3. Exercise Effect: Significant: On Day 5, in mice not administered PCB, IL-4 levels in exercised < not
exercised mice (*). In mice administered PCB 100, on Day 3, levels of IL-4 in exercised > not exercised animals (#). **PCB Effect: a) Significant:** In not exercised animals, on Day 5, levels of IL-4 in PCB 500 < PCB 0 administered mice ($). In mice not exercised, on Day 3, IL-4 levels in PCB 1000 < PCB 0 administered animals (£). On Day 5, in mice that were exercised, levels of IL-4 in PCB 1000 < PCB 0 administered animals (€). On Day 3, in mice that were exercised, IL-4 levels in PCB 500 < PCB 100 administered animals (¥). In mice that were exercised, on Day 3, levels of IL-4 in PCB 1000 < PCB 100 administered mice (&). **b) Trends:** On Day 3, in exercised mice, IL-4 levels in PCB 500 < PCB 0 administered animals. On Day 3, in animals that were exercised, levels of IL-4 in PCB 1000 < PCB 0 administered mice.
Fig. A-9: Effect of Exercise and PCB (Aroclor 1254) on levels of IL-5 in wound tissue at

Day 3 and Day 5 post wounding: Univariate ANOVA revealed significant day effect, significant PCB X day interaction and significant PCB X exercise X day interaction. Independent t-tests revealed the following within group interactions. Significance was determined as $p \leq 0.05$ and trend (not indicated in the figure) was determined as $p = 0.051$ to 0.1 ($n = 3$-6 mice). Day Effect: Significant: In exercised mice, administered PCB 100, levels of IL-5 on Day 5 < Day 3(*). In not exercised mice administered PCB 1000, IL-5 levels on Day 5 < Day 3(#). Exercise Effect: a) Significant: On Day 5, in mice administered PCB 1000, levels of IL-5 in exercised > not exercised animals(!). b) Trends: On Day 3, in mice administered PCB 100, IL-5 levels in
exercised > not exercised mice. In mice administered PCB 1000, on Day 3, levels of IL-5 in exercised < not exercised animals. **PCB Effect:**

* a) **Significant:** In exercised animals, on Day 3, levels of IL-5 in PCB 100 > PCB 0 administered mice (£). In mice that were exercised, on Day 3, IL-5 levels in PCB 500 < PCB 100 administered animals (€). On Day 3, in exercised animals, levels of IL-5 in PCB 1000 < PCB 100 administered mice (¥). In mice that were exercised, on Day 5, IL-5 levels in PCB 1000 > PCB 100 administered animals (§). In animals that were not exercised, on Day 5, levels of IL-5 in PCB 1000 < PCB 500 administered mice (¶). 

* b) **Trends:** On Day 5, in not exercised mice, levels of IL-5 in PCB 500 > PCB 100 administered animals. On Day 3, in mice that were not exercised, IL-5 levels in PCB 1000 > PCB 500 administered animals.
Fig. A-10: Effect of Exercise and PCB (Aroclor 1254) on levels of IL-10 in wound tissue at Day 3 and Day 5 post wounding: Univariate ANOVA showed significant PCB effect, significant day effect and significant exercise X day interaction. Independent t-tests revealed the following within group interactions. Significance was determined as $p \leq 0.05$ and trend (not indicated in the figure) was determined as $0.051 \leq p \leq 0.1$ (n = 3-6 mice). **Day Effect: a)**

*Significant:* In mice that were exercised and administered PCB 500, levels of IL-10 on Day 5 < Day 3(*). In exercised mice, administered PCB 1000, IL-10 levels on Day 5 < Day 3(#). **b)**

*Trends:* In mice that were not exercised and not administered PCB, levels of IL-10 on Day 5 < Day 3. **Exercise Effect: Significant:** On Day 3, in mice not administered PCB, IL-10 levels in
exercised > not exercised mice ($). In mice administered PCB 500, on Day 5, levels of IL-10 in exercised < not exercised animals(!). **PCB Effect:** a) **Significant:** On Day 3, in exercised mice, IL-10 levels in PCB 500 < PCB 0 administered animals (£). In mice that were exercised, on Day 5, levels of IL-10 in PCB 500 < PCB 0 administered animals (£). On Day 3, in exercised animals, IL-10 levels in PCB 1000 < PCB 0 administered mice (¥). In exercised mice, on Day 5, levels of IL-10 in PCB 1000 < PCB 0 administered animals (¥). b) **Trends:** In exercised animals, on Day 3, IL-10 levels in PCB 100 < PCB 0 administered mice. On Day 5, in exercised mice, levels of IL-10 in PCB 500 < PCB 100 administered animals. In exercised mice, on Day 3, IL-10 levels in PCB 1000 > PCB 500 administered mice.
Fig. A-11: Effect of Exercise and PCB (Aroclor 1254) on levels of IL-12(p40) in wound tissue at Day 3 and Day 5 post wounding: Univariate ANOVA did not show any significant individual or interaction effects. Independent t-tests revealed the following within group interactions. Significance was determined as p ≤ 0.05 and trend (not indicated in the figure) was determined as p = 0.051 to 0.1 (n = 3-6 mice). **Day Effect:** *a) Significant:* In mice that were not exercised and not administered PCB, levels of IL-12(p40) on Day 5 > Day 3(*). **b) Trends:** In mice that were not exercised and administered PCB 500, IL-12(p40) levels on Day 5 > Day 3(). **Exercise Effect:** **Trends:** In mice administered PCB 500, on Day 3, IL-12(p40) levels in exercised > not exercised animals. **PCB Effect:** *a) Significant:* On Day 5, in exercised mice,
levels of IL-12(p40) in PCB 1000 < PCB 0 administered animals (#). b) Trends: In not exercised animals, on Day 5, IL-12(p40) levels in PCB 500 < PCB 0 administered mice.
Fig. A-12: Effect of Exercise and PCB (Aroclor 1254) on levels of IL-12(p70) in wound
tissue at Day 3 and Day 5 post wounding: Univariate ANOVA showed significant PCB effect
and significant day effect. Independent t-tests revealed the following within group interactions.
Significance was determined as p ≤ 0.05 and trend (not indicated in the figure) was determined
as p = 0.051 to 0.1 (n = 3-6 mice). **Day Effect: Trends:** In mice that were not exercised and
administered PCB 100, levels of IL-12(p70) on Day 5 > Day 3. **Exercise Effect:** No significant
differences or trends in levels of IL-12(p70) between exercise and not exercised animals. **PCB
Effect: Significant:** In exercised animals, on Day 3, IL-12(p70) levels in PCB 500 < PCB 0
administered mice (*). On Day 5, in exercised mice, levels of IL-12(p70) in PCB 500 < PCB 0
administered animals (#). In exercised mice, on Day 3, IL-12(p70) levels in PCB 1000 < PCB 0 administered animals ($$).
Fig. A-13: Effect of Exercise and PCB (Aroclor 1254) on levels of IL-13 in wound tissue at Day 3 and Day 5 post wounding: Univariate ANOVA revealed trend for PCB effect and significant exercise X day interaction. Independent t-tests revealed the following within group interactions. Significance was determined as p ≤ 0.05 and trend (not indicated in the figure) was determined as p = 0.051 to 0.1 (n = 3-6 mice). Day Effect: No significant differences or trends in levels of IL-13 between Day 3 and Day 5 animals. Exercise Effect: Trends: In mice not administered PCB, on Day 3, levels of IL-13 in exercised > not exercised animals. On Day 3, in mice administered PCB 100, IL-13 levels in exercised > not exercised animals. Levels of IL-13 in exercised < not exercised mice was observed on Day 5, in animals administered PCB 1000.
**PCB Effect:** *a) Significant:* In exercised animals, on Day 3, IL-13 levels in PCB 100 < PCB 0 administered mice (*). On Day 3, in exercised mice, levels of IL-13 in animals administered PCB 500 < PCB 0 administered animals (#). *b) Trends:* In mice that were exercised, on Day 3, IL-13 levels in PCB 500 < PCB 100 administered mice. On Day 3, in animals that were exercised, levels of IL-13 in PCB 1000 > PCB 500 administered mice.
Fig. A-14: Effect of Exercise and PCB (Aroclor 1254) on levels of IL-17A in wound tissue at Day 3 and Day 5 post wounding: Univariate ANOVA revealed significant PCB effect, significant for exercise effect, trend of PCB X day interaction and significant exercise X day interaction. Independent t-tests revealed the following within group interactions. Significance was determined as p ≤ 0.05 and trend (not indicated in the figure) was determined as p = 0.051 to 0.1 (n = 3-6 mice). Day Effect: Significant: In mice that were not exercised and administered PCB 100, levels of IL-17A on Day 5 > Day 3(*). In mice that were exercised and administered PCB 1000, IL-17A levels on Day 5 < Day 3(#). Exercise Effect: Significant: On Day 3, in mice administered PCB 100, levels of IL-17A in exercised > not exercised mice ($). In mice
administered PCB 1000, on Day 3, IL-17A levels in exercised > not exercised animals(†). **PCB Effect:** 

*a) Significant:* On Day 3, in not exercised mice, levels of IL-17A in PCB 500 < PCB 0 administered animals (ը). On Day 3, in exercised animals, IL-17A levels in PCB 500 < PCB 0 administered mice (€). In mice that were not exercised, on Day 3, levels of IL-17A in PCB 1000 < PCB 0 administered animals (¥). On Day 5, in mice that were not exercised, IL-17A levels in PCB 1000 < PCB 0 administered animals (¶). In exercised animals, on Day 3, levels of IL-17A in PCB 500 < PCB 100 administered mice (¶). In exercised mice, on Day 5, IL-17A levels in PCB 500 < PCB 100 administered animals (✧). In not exercised animals, on Day 5, levels of IL-17A in PCB 1000 < PCB 100 administered mice (≈). In exercised mice, on Day 5, IL-17A levels in PCB 1000 < PCB 100 administered animals (※). In not exercised animals, on Day 3, levels of IL-17A in PCB 100 < PCB 0 administered mice. In mice not exercised, on Day 5, IL-17A levels in PCB 500 < PCB 0 administered animals. On Day 5, in mice that were not exercised, levels of IL-17A in PCB 500 < PCB 100 administered mice. In exercised mice, on Day 3, IL-17A levels in PCB 1000 > PCB 500 administered animals.
Fig. A-15: Effect of Exercise and PCB (Aroclor 1254) on levels of MIP-1α in wound tissue at Day 3 and Day 5 post wounding: Univariate ANOVA showed trend in exercise X day interaction and significant PCB X exercise X day interaction. Independent t-tests revealed the following within group interactions. Significance was determined as p ≤ 0.05 and trend (not indicated in the figure) was determined as p = 0.051 to 0.1 (n = 3-6 mice). **Day Effect: a)**

*Significant:* In mice that were not exercised and administered PCB 500, levels of MIP-1α on Day 5 > Day 3(*). **b) Trends:** In mice that were exercised and administered PCB 500, MIP-1α levels on Day 5 < Day 3. **Exercise Effect: a)** *Significant:* On Day 3, in mice administered PCB 500, levels of MIP-1α in exercised > not exercised mice (#). On Day 5, in mice administered PCB
500, MIP-1α levels in exercised < not exercised animals(I). b) *Trends:* In mice administered PCB 100, on Day 3, levels of MIP-1α in exercised > not exercised animals. **PCB Effect:** a)

*Significant:* In exercised animals, on Day 3, MIP-1α levels in PCB 100 > PCB 0 administered mice (£). On Day 5, in not exercised mice, levels of MIP-1α in PCB 500 > PCB 0 administered animals (€). On Day 5, in not exercised mice, MIP-1α levels in PCB 1000 < PCB 500 administered animals (§). b) *Trends:* In animals that were exercised, on Day 3, levels of MIP-1α in PCB 500 > PCB 0 administered mice. On Day 3, in exercised animals, MIP-1α levels in PCB 1000 > PCB 0 administered mice. In mice that were not exercised, on Day 5, levels of MIP-1α in PCB 1000 < PCB 100 administered animals.
Fig. A-16: Effect of Exercise and PCB (Aroclor 1254) on levels of MIP-1β in wound tissue at Day 3 and Day 5 post wounding: Univariate ANOVA showed significant PCB X exercise interaction and significant exercise X day interaction. Independent t-tests revealed the following within group interactions. Significance was determined as $p \leq 0.05$ and trend (not indicated in the figure) was determined as $p = 0.051$ to 0.1 ($n = 3$-$6$ mice). **Day Effect:** No significant differences or trends in levels of MIP-1β between Day 3 and Day 5. **Exercise Effect:** Significant: On Day 3, in mice not administered PCB, levels of MIP-1β in exercised $>$ not exercised mice ($^*$). In mice not administered PCB, on Day 5, MIP-1β levels in exercised $>$ not exercised animals ($^#$). **PCB Effect: a) Significant:** In mice not exercised, on Day 5, levels of MIP-1β in PCB 500 $>$ PCB 0
administered animals ($). b) Trends: In not exercised animals, on Day 5, MIP-1β levels in PCB 100 > PCB 0 administered mice. On Day 3, in exercised mice, levels of MIP-1β in animals administered PCB 100 < PCB 0 administered animals. On Day 3, in animals that were exercised, MIP-1β levels in PCB 500 < PCB 0 administered mice. In mice that were not exercised, on Day 5, levels of MIP-1β in PCB 1000 > PCB 0 administered animals.
Fig. A-17: Effect of Exercise and PCB (Aroclor 1254) on levels of RANTES in wound tissue at Day 3 and Day 5 post wounding: Univariate ANOVA showed significant PCB effect, significant exercise effect, trend in PCB X day interaction, significant exercise X day interaction and significant PCB X exercise X day interaction. Independent t-tests revealed the following within group interactions. Significance was determined as $p \leq 0.05$ and trend (not indicated in the figure) was determined as $p = 0.051$ to 0.1 ($n = 3$-$6$ mice). **Day Effect: Significant**: In mice that were exercised and not administered PCB, levels of RANTES on Day 5 < Day 3(*). **Exercise Effect: Significant**: On Day 3, in mice not administered PCB, levels of RANTES in exercised > not exercised mice (#). In mice administered PCB 1000, on Day 3, RANTES levels in exercised
PCB Effect: Significant: In exercised animals, on Day 3, levels of RANTES in PCB 100 < PCB 0 administered mice (†). On Day 3, in exercised mice, RANTES levels in PCB 500 < PCB 0 administered animals (€). In exercised mice, on Day 3, levels of RANTES in PCB 1000 < PCB 0 administered animals (€).