THE EFFECTS OF PLASTICIZER TREATMENT ON INFLAMMATION AND WOUND HEALING

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by

Irma Zia

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ABSTRACT

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Plasticizers are man-made chemicals used in the manufacture of a variety of products to ensure flexibility and longevity. While these compounds allow for the formation of more durable products, they have proven to be detrimental to the health of living organisms. Dibutyl phthalate (DBP) and bisphenol A (BPA) are two widely used plasticizers that are classified as endocrine-disrupting chemicals (EDC) due to their effects on developmental, endocrinological, reproductive, and metabolic function. We sought to investigate the effect of these compounds on the inflammatory response *in vitro* using a mouse macrophage cell line (RAW 264.7). Additionally, we used a live animal model (C57BL/6J mice) to determine the effects of DBP on the wound healing response. These studies indicated that high doses of either DBP or BPA altered the total cell count with time with or without exposure to bacterial lipopolysaccharide (LPS). When used in combination, an even greater effect was seen. We also found that treatment with these compounds, alone and/or in conjunction, had a significant effect on the measurable level of the cytokines interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) after LPS-treatment in RAW 264.7 cells. However, DBP did not affect wound healing in a mouse model, although there was a marked increase in the circulating level of TGF-β treated with 100 mg DBP/kg body weight. Overall, these data suggest that plasticizers can influence the inflammatory response which may have implications for human health.

KEY WORDS: Dibutyl phthalate, Bisphenol A, Inflammation, Plasticizers, Lipopolysaccharide, Wound healing, Endocrine disruptors

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CHAPTER I

Introduction

The impact of manmade products on the health and well-being of humans and animals alike is immeasurable. While many products have benefitted humans greatly due to the many advances they have made toward technology and modern medicine, there are just as many that have proven detrimental to humans and animals alike and the environment that surrounds them. From pesticides to chemicals found in everyday household products like cosmetics, toys and food containers, these products have the potential to cause harmful effects in humans; these include cancer and a plethora of developmental, endocrinological, and metabolic disorders. There is still much to be learned about the effects of many of these compounds on physiological function.

Phthalate Esters

Phthalate esters are organic compounds used as plasticizers in the manufacture of a variety of materials (Gupta 2017). Collectively, plasticizers are substances used to increase flexibility and prevent breakage in a variety of products during the manufacturing process (Hansen et al. 2015). For example, phthalates are added to polyvinyl chloride (PVC), a synthetic plastic polymer, to increase its durability, flexibility, and longevity (Gupta 2017). In addition, low molecular weight phthalates (<300 g/mol) (NRC 2008), such as dibutyl phthalate (DBP), are used as a solvent in many cosmetics and skincare products including lotions, perfumes, and nail polish (Gupta 2017). These organic compounds can also be found in pharmaceuticals, plastic food packaging items, and medical tubing and bags used for intravenous fluids, blood

transfusions, or dialysis (Li et al. 2013). Importantly, phthalates are not covalently bonded within the final product and are readily released into the surrounding environment with changes in temperature, pH, or the presence of high lipid content in the media since phthalates are lipophilic (Hansen et al. 2015; Gupta 2017).

Potential pathways of exposure to phthalates, as well as other plasticizers, include ingestion from food and water sources, inhalation from air and dust particles, direct intravenous exposure from medical tubing, and dermal exposure from cosmetics and skincare products (Li et al. 2013). While metabolic transformation via hepatic detoxification pathways result in their rapid excretion, the constancy of exposure to phthalates in the environment ensures that no individual is free of them at any time (Genuis 2012). Not surprisingly, DBP metabolites have been detected in the urine of over 90% of women and children tested during 2013-2014 (CDC 2015).

Many studies have documented the reproductive, endocrinological, and developmental effects of DBP in animal models, leading to its classification as an endocrine disrupting chemical (EDC) or endocrine disruptor (ED). An EDC/ED is any substance that can interfere with normal endocrinological pathways by mimicking, and thus competing with, naturally occurring hormones for their respective receptors (Hansen et al. 2015). For example, exposure to high levels of DBP during development can lead to hypospadias, undescended testes, and other testicular abnormalities due to its interference with androgen-dependent signaling (Mylchreest et al. 1998). Other studies have suggested that exposure to DBP can also lead to hepatomegaly, hypocholesterolemia, and hypotriglyceridemia because it is a known peroxisome proliferator and can thus interfere with peroxisome proliferator-activated receptor (PPAR) mediated signaling (Marsman

1995: Li et al. 2013). Recently, DBP has also been implicated as a carcinogen (Li et al. 2013; Gupta 2017) and an effect on immune function has also been documented. Specifically, an *in vitro* study using primary murine macrophages found that DBP exposure resulted in a decrease in cytokine production in response to LPS, as well as a decline in immunogenicity, or the ability to initiate an immune response (Li et al. 2013).

Bisphenols

Bisphenols are organic solvents that are important precursors to many plastic products, such as polycarbonates and epoxy resins used in common household products like Tupperware containers and food and beverage cans (Wetherill et al. 2007). The most widely studied bisphenol is bisphenol A or BPA. As is the case with most plasticizers, BPA can be released into the surrounding environment with a change in temperature or pH. Almost 90% of all BPA exposure can be traced to food products, while exposure via dermal contact and inhalation of dust particles account for about 5% (Acconcia et al. 2015).

BPA has also been classified as an EDC due to its ability to bind to the estrogen receptors; both estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) are targets of BPA. As with all classic steroid receptors, ERα and ERβ are transcription factors that change conformation and travel into the nucleus to regulate gene expression after binding to their naturally occurring first messenger, 17β-estradiol (E2). Interestingly, BPA is capable of acting as both a receptor agonist, a molecule that binds to and activates a receptor, and a receptor antagonist, a molecule that binds to and blocks a receptor's activity in a tissue-specific manner (Acconcia et al. 2015). Similar to DBP,

exposure to BPA can lead to a variety of developmental, metabolic, and reproductive disorders. For example, BPA causes infertility in both males and females by disrupting spermatogenesis and playing a role in the pathogenesis of polycystic ovarian syndrome (PCOS) (Konieczna et al. 2015).

The effects of BPA on immune function have also been studied. For example, low doses of BPA have been shown to non-specifically activate macrophages, thereby increasing the expression of the pro-inflammatory cytokines, interleukin 4 (IL-4), interleukin 5 (IL-5), and interleukin 13 (IL-13) (Loffredo et al. 2020). Normally, macrophages will differentiate into different subclasses with unique biological functions in response to specific environmental stimuli, such as the presence of antigens (i.e. substances that are recognized as foreign by the host cell) or inflammatory mediators, in a process called macrophage activation or polarization. Nonspecific macrophage activation, however, occurs in the presence of substances that are not recognized as foreign by the host cell and do not initiate an inflammatory response (Mosser and Edwards 2008; Yao et al. 2019).

In another study, the LPS-induced expression of TNF-α was decreased in both peritoneal macrophages isolated from 5-7-week-old female mice (free-BALB/C) and RAW 264.7 cells when exposed to high concentrations of BPA (Kim and Jeong 2003). While there have been many studies involving the effects of BPA on different aspects of the immune response, research documenting the effects of BPA on macrophages is especially important, as macrophages are one of the most important components of the inflammatory response and provide the first line of defense against potential pathogens (Kumar 2020).

The Inflammatory Response

The inflammatory response, commonly known as inflammation, plays a crucial role in innate immunity and the wound healing process. More specifically, inflammation is the body's first response to an infection, irritation (an irritant is any non-infectious substance that can cause an immune response), or tissue damage (Henry and Garner 2003), and a proper inflammatory response is imperative for overall well-being. For example, overactive or chronic inflammation can lead to autoimmune disease and cancer (Coussens and Werb 2002; Freire and Dyke 2013; Ahmed and Kaveri 2018), while underactive and/or suppressed inflammation can lead to a variety of persistent infections (Ahmed and Kaveri 2018). Inflammation is usually characterized by the presence of redness, heat, swelling, and pain. Sometimes, a loss of function in the affected area is also seen (Freire and Dyke 2013).

Pathogen Associated Molecular Patterns (PAMPs) are molecular signatures such as cell wall components that are recognized as foreign by host cells to induce an inflammatory response. These signatures can either be shared among groups of microorganisms (e.g. bacteria) or they can be unique to a specific microorganism (Takeuchi and Akira 2010). One of the best studied PAMPs is bacterial lipopolysaccharide (LPS), an endotoxin found in the cell wall of gram-negative bacteria. Structurally, LPS is composed primarily of three domains: the o antigen, the lipid A region, and the core oligosaccharide. The host immune system will recognize and respond primarily to the lipid A region, which is highly conserved among species. However, because of variation within the structure of LPS among strains, even within the

lipid A region, different species can initiate different levels of response by the host (Bertani and Ruiz 2018).

The recognition of PAMPs by the host leads to the secretion of inflammatory mediators, such as cytokines, by resident cells. Cytokines are signaling proteins with pleiotropic properties; that is, the effect of a given cytokine can vary from one cell type to another (Henry and Garner 2003; Takeuchi and Akira 2010). Overall, cytokines regulate tissue death, the degree of vascular permeability, and play an essential role in the recruitment of cells, such as macrophages, to the inflamed area (Takeuchi and Akira 2010). In some cases, the final outcome mediated by a cytokine can be antagonistic depending on context, e.g. cell location, cell type, *et cetera.* For example, interleukin 6 (IL-6) plays a prominent role in the inflammatory response by initiating the acute phase response and attracting monocytes to the area of inflammation. However, IL-6 can also contribute to the resolution of the inflammatory response by inducing apoptosis of cells key to inflammation, such as neutrophils (Scheller et al. 2011).

Likewise, Damage Associated Molecular Patterns (DAMPs), which are endogenous substances such as DNA, ATP, or heat-shock proteins released by damaged and/or dying cells, can alert the host immune response to a potential threat. DAMPs are recognized in the same manner as PAMPs, resulting in an inflammatory response (Roh and Sohn 2018).

Pattern recognition receptors (PRRs) are a diverse group of proteins that identify and bind to both PAMPs and DAMPs. To date, four different classes of PRRs have been identified: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and NOD-like receptors (NLRs) (Takeuchi

and Akira 2010). These PRRs are distributed ubiquitously in an intact organism and exist as either transmembrane receptors to bind extracellular targets or within the cell, i.e. in the cytoplasm or in an endolysosome, to bind intracellular targets (Takeuchi and Akira 2010). RLRs and NLRs can recognize PAMPs from viruses and bacteria respectively as well as certain DAMPs, while CLRs are able to recognize PAMPs from fungi and specific DAMPs. TLRs have a much broader recognition range, which includes DAMPs and PAMPs from viruses, bacteria, parasites, and protozoa (Takeuchi and Akira 2010).

Once a PRR is bound by its specific ligand, a signaling cascade is activated that modulates the expression of genes involved in different aspects of an inflammatory response, such as the secretion of cytokines and interferons. Moreover, the specific inflammatory response that is activated is dependent on the specific PAMP and/or DAMP. For example, toll-like receptor 4 (TLR-4) is a transmembrane receptor protein that is widely known for recognizing LPS. More specifically, LPS is recognized by TLR-4 in conjunction with CD14 (cluster of differentiation 14) and MD2 (lymphocyte antigen 96) (Copeland et al. 2005; Park and Lee 2013). This TLR-4/CD14/MD2 receptor complex can initiate two distinct signaling cascades that ultimately lead to the activation of the transcription factors nuclear factor kappa B (NF-κB), activator protein 1 (AP1), and interferon regulatory factor 3 (IRF3). Which signaling cascade is activated depends on the adaptor protein, MyD88 or TRIF, that is recruited to the receptor complex. MyD88 is recruited to the receptor complex at the cell surface, and this ultimately leads to the activation of NF-κB and AP1. Both of these transcription factors are responsible for the upregulation of a suite of pro-inflammatory genes resulting in the production and secretion of inflammatory mediators, mainly pro-inflammatory cytokines (Park and Lee

2013). This is called the MyD88-dependent pathway (Figure 1) and is characterized by the release of specific cytokines. In response to LPS, tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and interleukin 6 (IL-6) are the predominant cytokines released. While other cytokines, like IL-8, can also be released in response to LPS, in this study we chose to focus on TNF-α, IL-6, and IL-1β (Levy 1996; Möller and Villiger 2006; Takeuchi and Akira 2010).

TNF-α is mainly responsible for regulating other immune cells: it can upregulate the secretion of other pro-inflammatory cytokines by further activating the NF-κβ pathway, and it can attract neutrophils to the site of infection. TNF-α can also increase the killing ability of macrophages by stimulating phagocytosis. The major symptoms of inflammation, including redness, heat, swelling, and pain, are usually caused by a localized increase of TNF-α (Parameswaran and Patial 2010).

When IL-6 is secreted, it acts on the liver to initiate the acute phase response characterized by the release of acute phase proteins, such as C-reactive protein and serum amyloid A, into the circulation (Kaur et al. 2020). Acute phase proteins play a role in the febrile response, as well as attracting neutrophils and leukocytes to the site of infection. They can also enhance phagocytosis by marking specific cells, in a process known as opsonization, to be recognized by macrophages and natural killer cells (Jain et al. 2011).

While upregulation of the MyD88-dependent pathway can cause the secretion of IL-1β, it is not in its active or mature form. This form, termed pro-IL-1β, requires cleavage by caspase 1 before it can bind to IL-1 receptors. Caspase 1 is a protease, an enzyme capable of cleaving other proteins, but it must be activated itself through the

formation of the inflammasome. IL-1β can then promote the secretion of acute phase proteins and the febrile response (Zheng et al. 1995; Dinarello 2017).

Once the MyD88-dependent pathway has been initiated, the receptor complex is endocytosed off the cell surface into an endosome. This is where TRIF is recruited to the receptor complex, and this activates a different pathway, termed the MyD88-independent pathway or the TRIF-dependent pathway (Figure 1). The downstream signaling cascade in this pathway leads to the activation of IRF_3, which is responsible for the expression of type I interferon (IFN) genes, thereby upregulating the secretion of type 1 interferons. Interestingly, there is also crosstalk between the MyD88-dependent and TRIF-dependent pathways, such that TRIF can also upregulate both NF-κB and AP1, thus further inducing pro-inflammatory gene expression (Ullah et al. 2016).

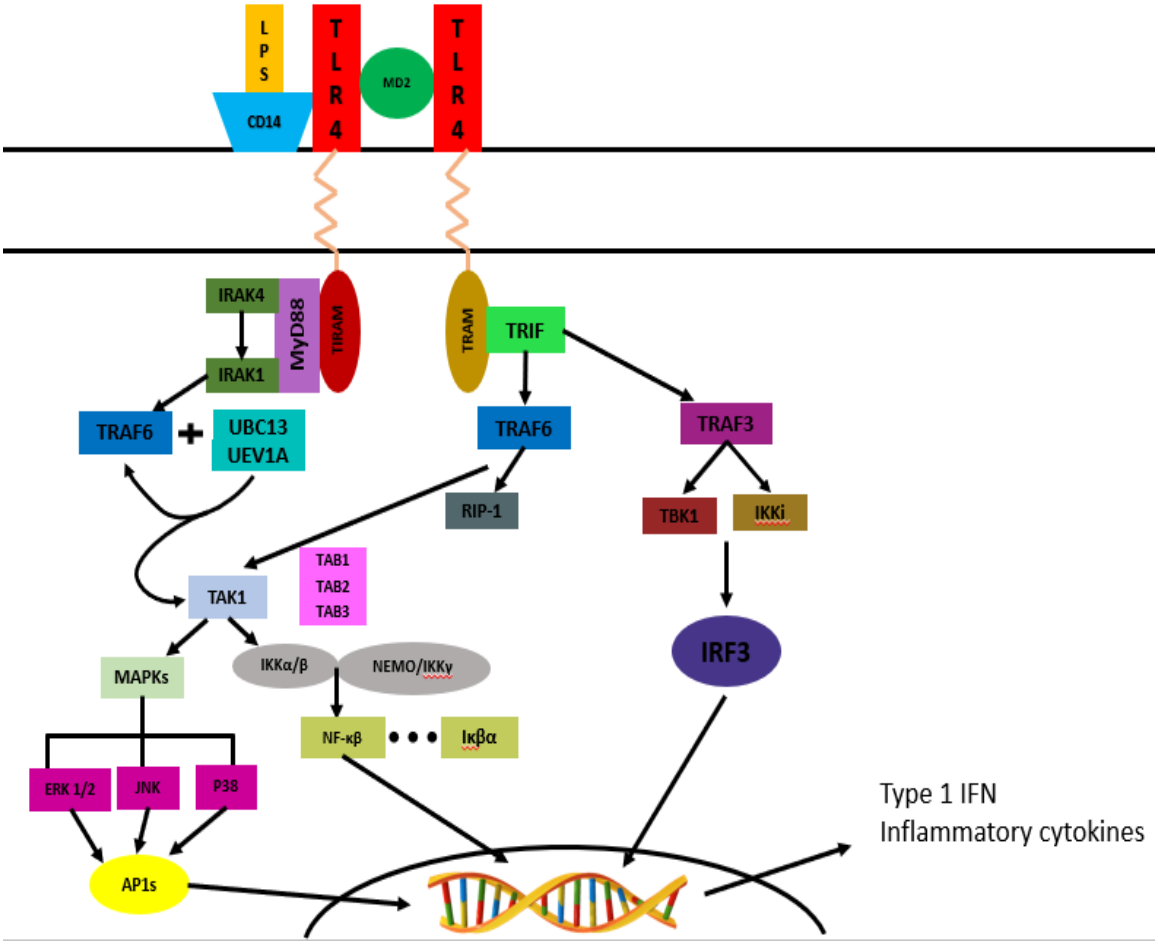

Figure 1.MyD88-dependent & TRIF-dependent Signaling. TIRAP recruits MyD88 to the cell surface. MyD88 forms a complex with the kinases IRAK 4 and IRAK1. IRAK 4 activates IRAK1, causing it to autophosphorylate and be released from MyD88. IRAK1 then associates with TRAF6. TRAF6 promotes the polyubiquitination of itself and of the TAK1 protein complex. TAK1 forms a complex with TAB1, TAB2, and TAB3, which can interact with the polyubiquitin chains formed by TRAF6 to activate TAK1. TAK1 can then activate the MAPK and NF-κB pathways. TAK1 binds to the IKK complex and activates IKKβ. The IKK complex then phosphorylates Iκβα, which is an inhibitory protein for NF-κβ. Iκβα is then degraded by the proteasome, allowing NF- κβ to translocate into the nucleus to induce proinflammatory gene expression. TAK1 can also activate MAP3K family members, ERK1/2, p38, and JNK. This allows for the activation of the AP-1 family of transcription factors to further regulate inflammatory responses. The TRIF-dependent pathway induces the production of both type 1 interferons and proinflammatory cytokines. TRAM is recruited to an endosome to act as a link between TLR4 and TRIF. TRIF then interacts with TRAF6 to recruit RIP-1, a protein kinase, which activates the TAK1 complex, allowing for the activation of the MAPK and NF-κB pathways. TRIF also interacts with TRAF3 to recruit TBK1 and IKKi, which activate IRF_3. IRF_3 then forms a dimer and translocates into the nucleus to induce the expression of type 1 interferon genes. (Adapted from: Kawaski and Kawai 2014; Ullah et al. 2016).

Wound Healing

When tissue damage has occurred, a wound healing response is initiated. This consists of four distinct, but overlapping, phases known as hemostasis, inflammation, proliferation, and wound maturation (Figure 2) (Henry and Garner 2003). Hemostasis is the process of stopping the flow of blood. This is achieved by the constriction of blood vessels and the initiation of the coagulation cascade (Henry and Garner 2003). Inflammation, of course, is induced to combat any resultant infection by removing foreign microorganisms and damaged tissue.

The proliferative phase is characterized by fibroplasia, the formation of new fibrous tissue, and angiogenesis, the formation of new blood vessels, so that the newly formed tissue can receive proper oxygen and nutrients (Kirsner and Eaglstein 1993; Henry and Garner 2003). Wound contraction, which is necessary for closure of the wound, occurs in the late stages of the proliferative phase (Kirsner and Eaglstein 1993; Henry and Garner 2003). Wound maturation, also called wound remodeling, will begin roughly 21 days after wounding. This stage is characterized by a constant amount of collagen present in the wound, which is important for recreating the pre-wounded structure (Henry and Garner 2003)

In reality, inflammation persists throughout the wound healing process since aspects of each stage involve inflammatory mediators. For example, inflammatory mediators that are typically induced by an LPS challenge (TNF-α, IL-1β, IL-6) are also present at the wound site during the wound healing process. TNF-α is necessary for recruiting neutrophils to the wound area, as well as inducing the secretion of IL-8 which is central to re-epithelialization and activating angiogenesis. Interlekin-1 (IL-1) is also

involved in re-epithelialization and angiogenesis, as well as helping to stimulate fibroblasts for the formation of new tissue. IL-6 inhibits the breakdown of the extracellular matrix formed during the proliferative phase. In addition, transforming growth factor beta (TGF-β) stimulates hemostasis, inflammation, and the production of collagen from fibroblasts in conjunction with IL-1 (Henry and Garner 2003).

In this study, we sought to investigate the effects of the plasticizing agents, DBP and BPA, on the level of pro-inflammatory cytokines (i.e. TNF-α, IL-6, and IL-1β) after exposure to LPS using the RAW 264.7 murine macrophage cell line. In addition, we wanted to determine if the combined effect of the two compounds was any different from when the compounds were used individually. While several studies have documented the effects of either DBP or BPA alone on the inflammatory response, few have determined whether there is a synergistic effect when the two are used in conjunction. Additionally, since inflammatory mediators are prevalent in the regulation of wound healing, we sought to investigate the effects of DBP on the wound healing process in C57BL/6 mice.

Null Hypothesis #1: Neither DBP nor BPA alone will have an effect on the level of TNF-α, IL-6, and IL-1β after LPS exposure in RAW 264.7 cells.

Null Hypothesis # 2: The effect of the two compounds in combination on cytokine level will not be any different from the effect seen when the two compounds are used individually.

Null Hypothesis #3: DBP will not alter the wound healing response in C57BL/6J mice.

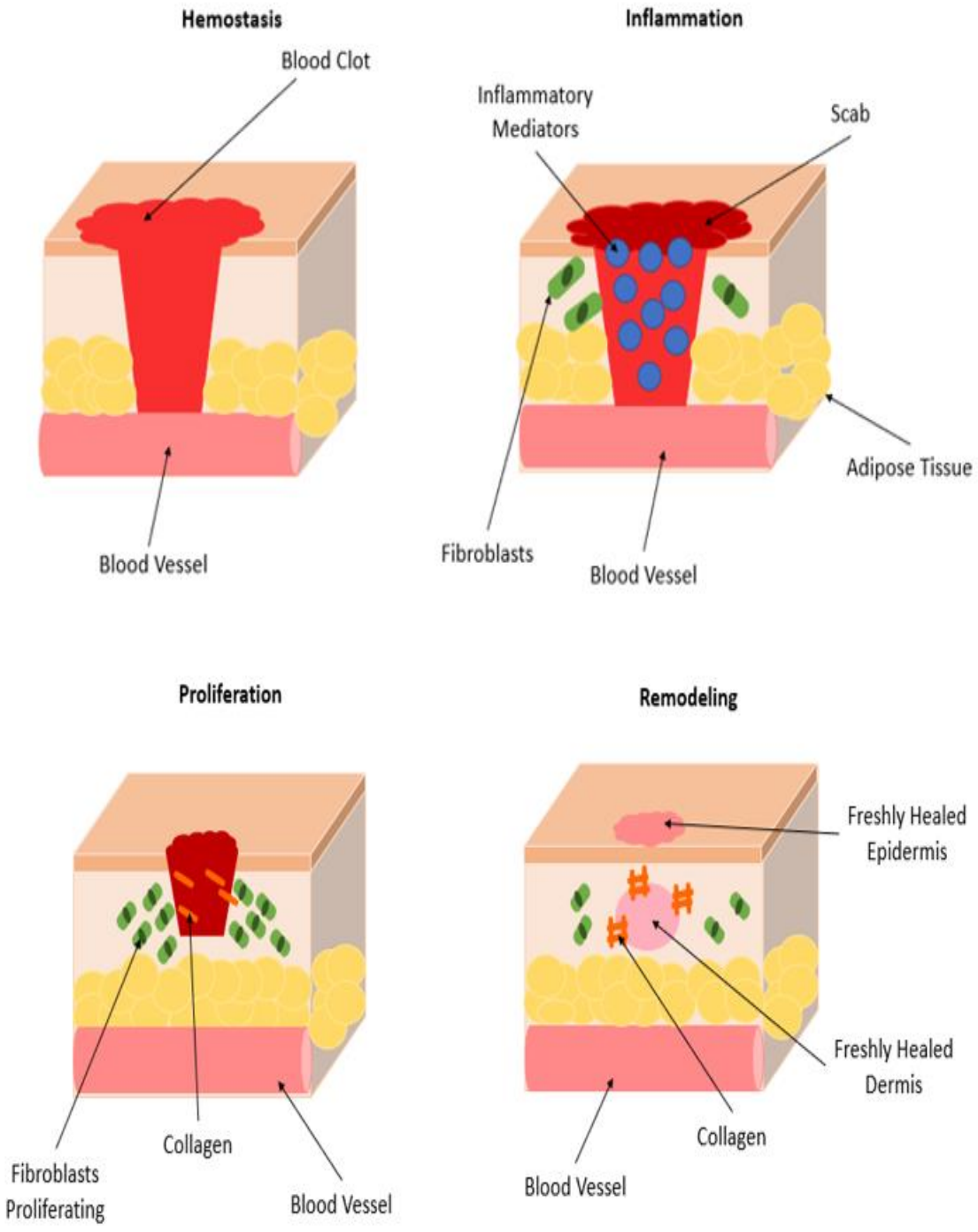

Figure 2. The Wound Healing Process.
(Adapted from: https://www.inovanewsroom.org/ilh/2017/05/wound-healing-center-at-inova-loudoun-treats-complex-wound-and-ostomy-cases/)

CHAPTER II

Methods

Cell Line

The RAW 264.7 murine macrophage cell line, originally from the American Type Culture Collection (ATCC) and gifted to us by Dr. Jeremy Bechelli at Sam Houston State University, was used for all *in vitro* experiments. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), with the addition of 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The effect of DBP and BPA on the total cell count, cell viability and LPS-induced secretory profiles of select cytokines was assessed using a 12-well tissue culture plate with cells seeded at a density of 5×10^5 cells per well in 1000 µL of media. The seeding density was determined using a guide provided by ThermoFisher Scientific (https://www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/cell-culture-useful-numbers.html).

Cell counts were estimated using a Countess Automated Cell Counter (ThermoFisher/Invitrogen) to determine the concentration of cells per mL of media. Afterward, the media was diluted appropriately to reach the desired density using growth media. In some cases, 50 µL of the cell suspension was mixed with an equal volume of trypan blue to determine the ratio of live–to-dead cells. After seeding, cells were allowed to adhere to the plate for 24 hours in an incubator set at 37℃ with 5% CO_2.

In Vitro Assessment of Total Cell Count & Viability after Exposure to DBP/BPA

The effect of DBP and BPA alone, as well as DBP in combination with BPA, on the cell count and viability was measured via trypan blue exposure as described above. In

a preliminary study (Results, Figures 5-8) the dose of each compound (Kim et al. 2013; Li et al. 2013) and an exposure time with minimal signs of apparent toxicity were determined. Specifically, approximately 24 hours after seeding, the growth media was removed and replaced with media containing either DBP or BPA at each of two doses (20 and 80 µM), as well as the compounds in combination which included: 20 µM DBP + 20 µM BPA, 80 µM DBP + 80 µM BPA, 20 µM DBP + 80 µM BPA, and 80 µM DBP + 20 µM BPA. Each of the wells were aspirated and washed once with 1x phosphate buffered saline (PBS) before the addition of the compounds. Both DBP and BPA were dissolved in 100% ethanol (EtOH) to generate a stock solution, then diluted in DMEM to reach the desired concentration in 1% EtOH. A media control, 1 ml of DMEM, and a vehicle control, 1 ml of 1% EtOH diluted in DMEM, were included in each experiment. To minimize the disturbance to the cells, individual plates were used for each exposure period. At 5 and 24 hours after exposure, cells were dislodged from the plate using a cell scraper and transferred to microcentrifuge tubes, then spun at 1000 RPM for 5 minutes to pellet the cells. The wells in the plate were observed using a compound microscope immediately after scraping to ensure that the maximum number of cells possible were being dislodged. The supernatant was decanted, and the pellet was resuspended in 50 µL of 1x PBS, and an equal volume of Trypan Blue to generate cell number and cell viability estimates using 10 µL of this mixture in the Countess. This experiment was repeated three times for each exposure period (Figure 3).

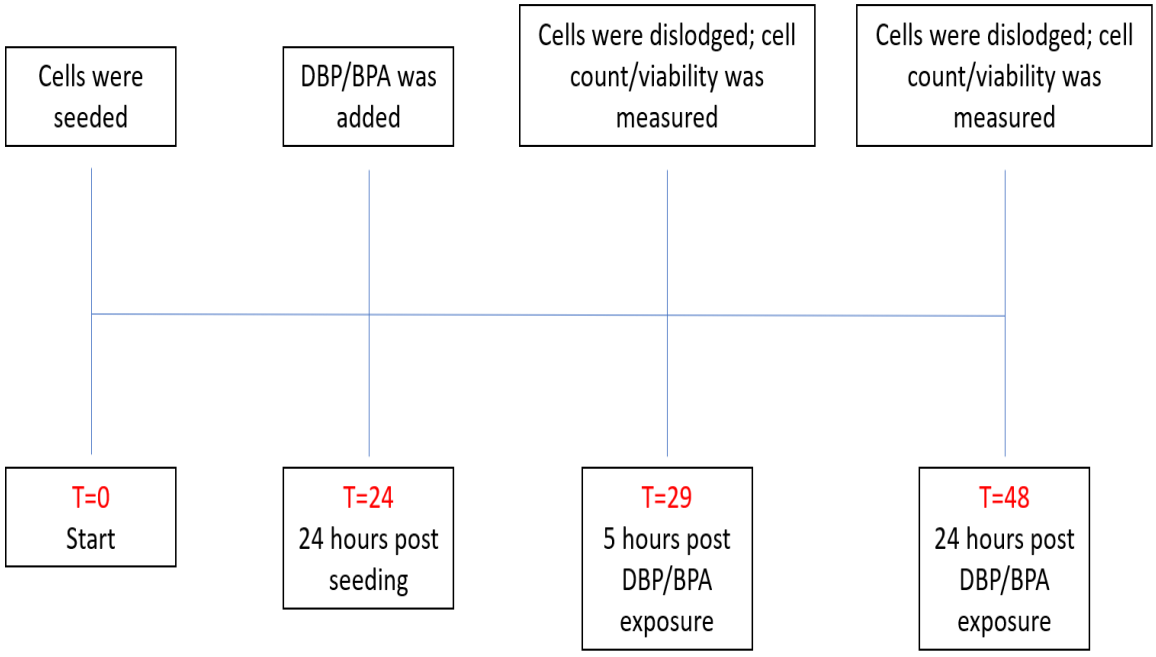

Figure 3. Timeline for Preliminary Study

***In Vitro* Assessment of Cell Count/Viability & Cytokine Profiles after LPS Exposure**

Based on the preliminary experiment described above, the final doses of the compounds used were unchanged and a 24-hour exposure was chosen as the amount of time the cells would be exposed to the compounds. As above, approximately 24 hours after seeding, each well of a 12-well plate was aspirated and washed with 1x PBS prior to the addition of DBP/BPA at the indicated doses (see above). Both DBP and BPA were dissolved in 100% ethanol (EtOH) as before. Twenty-four hours-post DBP/BPA exposure, the wells were aspirated and washed with 1x PBS, then exposed to LPS (*Escherichia coli* 055: B5; 1 µg/mL; dissolved in growth media) (Gaekwad et al 2010; Xiang et al. 2015). The cells were not exposed to LPS in conjunction with DBP/BPA in order to assess whether there was a lasting effect of the exposure to these plasticizers.

At 8, 24 and 48 hours the media from each well on an individual plate was collected and stored at -80℃ until used for the assessment of IL-6, IL-1β, and TNFα levels via enzyme immunoassay (EIA) according to the manufacturer's instructions (ThermoFisher/Invitrogen & TONBO Biosciences). In addition, after removing the media, 500 µL of 1x PBS was added to each well to collect the cells via scraping. Fifty µL of this solution was added to 50 µL of Trypan Blue, and 10 µL was used in the Countess to determine total cell count and viability as described above. The remainder of the cell suspension was used to prepare cell lysates, which were then stored at -80℃. The cell lysates were not used in this study. This procedure was repeated 5 times for each timepoint (Figure 4).

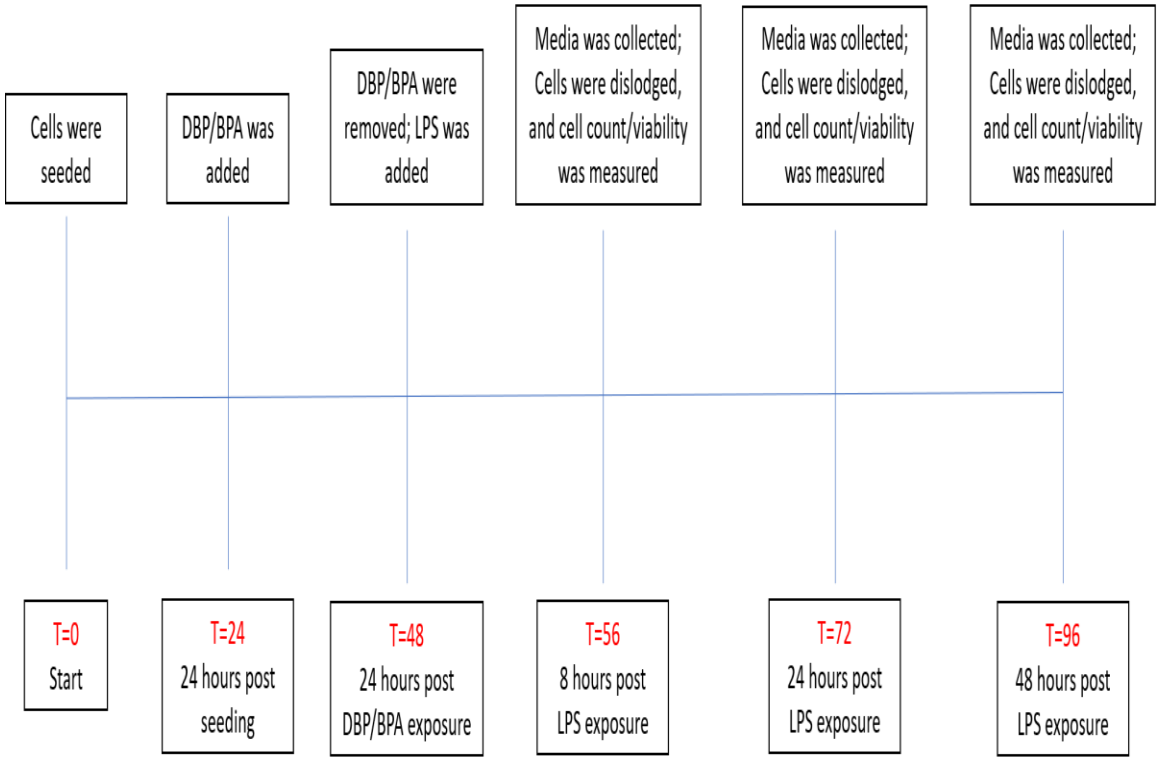

Figure 4.Timeline for DBP/BPA Study after LPS Exposure

In Vivo Effect of DBP on Wound Healing in Mice

Mice

This protocol was approved by IACUC (ID #18-10-22-1024-3-01)

A total of 54 male C57BL/6J mice aged 2-5 months were purchased from the Science Annex at Sam Houston State University. These mice were the descendants of breeder mice originally obtained from the Jackson Lab. The mice were housed in the Science Annex, with a 12-hour light/12-hour dark cycle, and food (Envigo Teklad 2018 18% protein rodent diet) and water provided *ad libitum*. A total of 3 trials was conducted (18 mice per trial) to determine whether chronic exposure to DBP affected the rate and/or the degree of wound healing using an ear hole punch model.

Wound Healing Assay

The study took place over a 28-day period. On day 1 of each trial, all of the mice were weighed, then randomly assigned to one of 4 groups using a random number generator: untreated control (n = 3), vehicle control (n = 5), low DBP treatment (n = 5), and high DBP treatment (n = 5). After the initiation of the experiment, individual mice were weighed weekly. In addition, the vehicle control and DBP treated mice were injected daily with either 50 µL of heat sterilized vegetable oil (vehicle control) or 50 µL of heat sterilized vegetable oil containing DBP such that mice received either 100 mg DBP/kg body mass (low treatment) or 500 mg DBP/kg body mass (high treatment) (Giribabu et al. 2012). The untreated control mice were left undisturbed except for weekly weighing, weekly blood collection, and assessment of wound healing (see below). Since each mouse received a mass specific dose of DBP delivered, the total amount of

DBP delivered to each mouse was adjusted in accordance with any change in body mass on a weekly basis. All injections were given intraperitoneally (i.p.) and continued for a total of 28 days.

Immediately prior to injection on day 1, a 2 mm hole punch was administered to every mouse's right ear using a 2 mm scissor-style ear punch (World Precision Instruments, LLC) and a picture was taken using a Canon PowerShot SX530 HS. Once a week (i.e. on Days 7, 14, 21 and 28), another picture was taken to assess the degree of wound healing. Using ImageJ software, the area of each of the ear holes was calculated over time using the freehand selection tool. More specifically, the outline of the entire right ear was traced, as well as the outline of the hole, and the area of each calculated to generate a relative wound size. In several instances, the wound opened past the margin of the ear, which made it impossible to quantify the wound healing response; these mice were removed from the study. In total, one untreated control (1/9), one vehicle control (1/15), 3 low DBP treated (3/15), and 4 high DBP treated (4/15) mice were excluded.

Blood samples (~100 µl) were also collected from all mice via submandibular bleeding at the time of weighing each week. The blood samples were allowed to clot for 30 minutes at room temperature before spinning for the isolation of serum. Serum samples were then stored at -80℃ until used for the assessment of TGF-β levels via EIA according to the manufacturer's instructions (Thermo Fisher/Invitrogen). On day 28 the mice were euthanized via CO_2 asphyxiation, and the heart, kidneys, spleen, and liver were harvested and stored at -80℃. The ears were also harvested and stored in formalin. These tissues were not analyzed as part of this study. During Trial 2, it was observed that one of the mice in the high treatment group had lost more than 20% of its body weight

and was acting lethargic. Since these were clear signs of illness, the mouse was euthanized to prevent further suffering.

Statistical Analysis

A multivariate analysis of variance (ANOVA) was used to analyze the data from all *in vitro* experiments, followed by Dunnett's *post hoc* test using the vehicle control group as the reference. A two-way repeated measures ANOVA was used to analyze the data obtained from the *in vivo* experiments and a Tukey's Test was used for *post hoc* analysis as appropriate. All statistical analyses were conducted using IBM SPSS version 26. A P-value of less than or equal to 0.05 was used to determine statistical significance.

CHAPTER III

Results

Plasticizer Treatment Alters Total Cell Count and Viability in RAW 264.7 Cells

In the preliminary study total cell count and viability were assessed after RAW 264.7 cells were exposed to DBP, BPA, or both for either 5 or 24 hours. This was done to ensure that the doses chosen were not outright toxic to the cells.

An increase in both cell count and viability was seen from the 5-hour timepoint to the 24-hour timepoint across all of the groups. For both total cell count and cell viability, none of the treatment groups were significantly different from the vehicle control at 5 hours post DBP/BPA exposure (Figures 5-8). At 24 hours, however, an effect was seen. The presence of the vehicle led to a decrease in the total number of cells relative to the media control (Figures 5 & 6). In addition, the presence of the high dose of BPA (80 µM) (Figure 5) and the presence of DBP and BPA in conjunction (all combinations) (Figure 6) further decreased the total number of cells; and this decrease was statistically significant when compared to the vehicle control. The results for cell viability were consistent with that of total cell count, except that the presence of the vehicle did not seem to have an effect (Figures 7 & 8).

These data suggest that exposing RAW 264.7 cells to BPA, alone and in combination with DBP, for 24 hours can lead to a decrease in total cell count and cell viability. While the decrease seen was significant relative to the vehicle control, it was not all that dramatic, and so the same initial doses were used going forward.

Plasticizer Treatment Alters Total Cell Count and Viability after Exposure to LPS

RAW 264.7 cells were exposed to DBP, BPA, or both for 24 hours and then subsequently exposed to LPS for either 8, 24, or 48 hours. Total cell count and cell viability were assessed at each of the timepoints after LPS exposure (Figures 9-12).

Both total cell count and viability peaked at 24 hours and then dropped dramatically at 48 hours. At the 8-hour timepoint, the presence of the high dose of DBP (80μM) (Figure 9) alone, the high dose of BPA (80μM) (Figure 9) alone, and both compounds in conjunction (all combinations) (Figure 10) led to a decrease in the total number of cells; and this decrease was statistically significant when compared to the vehicle control. A similar result was seen at 24 hours (Figures 9 & 10), except that the high dose of DBP (80μM) (Figure 9) did not have an effect. No effect was seen at the 48-hour timepoint (Figures 9 & 10).

At the 8-hour timepoint, when the compounds were used in conjunction (Only 2 combinations: DBP 80 μM + BPA 80 μM & DBP 80 μM + BPA 20 μM) (Figure 12) a significant decrease in viability was observed relative to the vehicle control. At 24 hours, all combinations of the two compounds led to a significant decrease in viability relative to the vehicle control, except when 20 μM of DBP was used in conjunction with 20 μM of BPA (Figure 12). At 48 hours, the presence of the high dose of DBP (80 μM) (Figure 11) alone led to a decrease in viability, while the presence of 80 μM of DBP used in conjunction with 80 μM of BPA (Figure 12) led to an increase in viability; these differences were statistically significant when compared to the vehicle control.

Overall, it was found that DBP and BPA, either alone or in conjunction, can decrease total cell count in RAW 264.7 cells after exposure to LPS.

Plasticizer Treatment Alters IL-6, TNF-α, and IL-1β Level after LPS Exposure

In addition to measuring total cell count and cell viability, cytokine profiles were assessed at 8, 24, and 48 hours after LPS exposure (Figures 13-18).

For IL-6, no effect was seen at the 8-hour timepoint (Figures 13 & 14). At 24 hours, IL-6 level was significantly decreased in the presence of all combinations of DBP and BPA relative to the vehicle control (Figure 14), except when 20 µM of DBP was used in conjunction with 20 µM of BPA. The presence of the high dose of DBP (80 µM) (Figure 13) alone and the presence of the two compounds used in conjunction (all combinations) (Figure 14) resulted in a decrease in IL-6 level at the 48-hour timepoint. These decreases were statistically significant when compared to the vehicle control.

In the case of TNF-α, only the presence of 80 µM of DBP used in conjunction with 80 µM of BPA resulted in a decrease at the 8 and 24-hour timepoints (Figure 16). These decreases were statistically significant when compared to the vehicle control. At 48 hours, the presence of 80 µM of DBP used in conjunction with 20 µM of BPA resulted in a significant increase in TNF-α level relative to the vehicle control (Figure 16).

There was no detectable IL-1β at 8 hours after LPS treatment for all treatment groups, although there was an appreciable level at both 24 and 48 hours (Figures 17 & 18). Unlike IL-6 or TNF-α, the presence of the vehicle led to a significant increase in IL-1β level after exposure to LPS relative to the media control at both 24 and 48 hours (Figures 17 & 18). At the 24-hour timepoint, cells exposed to 20 µM of DBP alone exhibited the greatest IL-1β level after LPS treatment (Figure 17). This increase was

statistically significant when compared to the vehicle control. Additionally at 24 hours, cells exposed to 80 µM of DBP alone (Figure 17) or to 20 µM of DBP in combination with 80 µM of BPA (Figure 18) had significantly decreased levels of IL-1β relative to the vehicle control.

There was no difference for each of the remaining treatments except that the cells exposed to 80 µM of DBP in combination with either 20 or 80 µM of BPA had dramatically lower IL-1β level (data not shown). Specifically, cells exposed to 80 µM of DBP in conjunction with 80 µM of BPA resulted in an undetectable level of IL-1β at both 24 and 48 hours, while 80 µM of DBP in conjunction with 20 µM of BPA resulted in a detectable level of IL-1β in only 40% (2/5) of the wells at 24 hours post-LPS exposure. Meanwhile, although 100% (5/5) of the wells exposed to 80 µM of DBP in conjunction with 20 µM of BPA had a detectable level of IL-1β at 48 hours post-LPS exposure, it was at a level that is at least 70% lower (10.26 ± 0.62 pg/mL) than all the other treatments (Figure 18).

These data suggest that both DBP and BPA can decrease IL-6 level after LPS exposure, and that this effect is even more pronounced when the two compounds are used in combination. While DBP alone and BPA alone do not alter TNF-α level after exposure to LPS, when the high doses of both compounds are used in conjunction, a decrease was seen. The presence of DBP, alone and especially in conjunction with BPA, altered the level of IL-1β (usually a decrease was seen) after exposure to LPS.

Chronic DBP Treatment does not Influence Overall Wound Closure

Chronic treatment with DBP had no effect on body mass over the course of the four-week trial, as there was no significant difference in the mean body mass among the groups both within and between weeks (data not shown). Daily handling and injections did not influence body mass either.

Prior to analysis, the relative size of the wound was set to 1 for each of the treatment groups at Week 1 post-wounding. As expected, relative wound size declined with time for each of the groups of mice (Figure 19A). As can be seen from the slopes of the lines in Figure 19A, the rate of healing was not dramatically different.

Additionally, overall wound closure was also assessed (Figure 19B). Despite the degree of healing appearing to be different among the groups when the total percent closure was estimated after four weeks, this difference was not statistically significant.

Overall, DBP did not influence the wound healing response using this *in vivo* model.

Chronic Treatment with Low Concentration of DBP Leads to an Increase in Systemic TGF-β Level

In addition to monitoring wound healing, the level of systemic TGF-β was assessed from the weekly blood samples that were collected (Figure 20). Overall, systemic TGF-β level gradually increased from week 0 to week 4 across all treatments, and those mice treated with the low dose of DBP had the highest level of TGF-β. This level was significantly different relative to the untreated control group only. None of the other groups were different.

These data suggest that systemic TGF-β level increases in response to tissue damage *in vivo.* In addition, the presence of the low dose of DBP (100 mg DBP/kg body weight) increases circulating TGF-β even further.

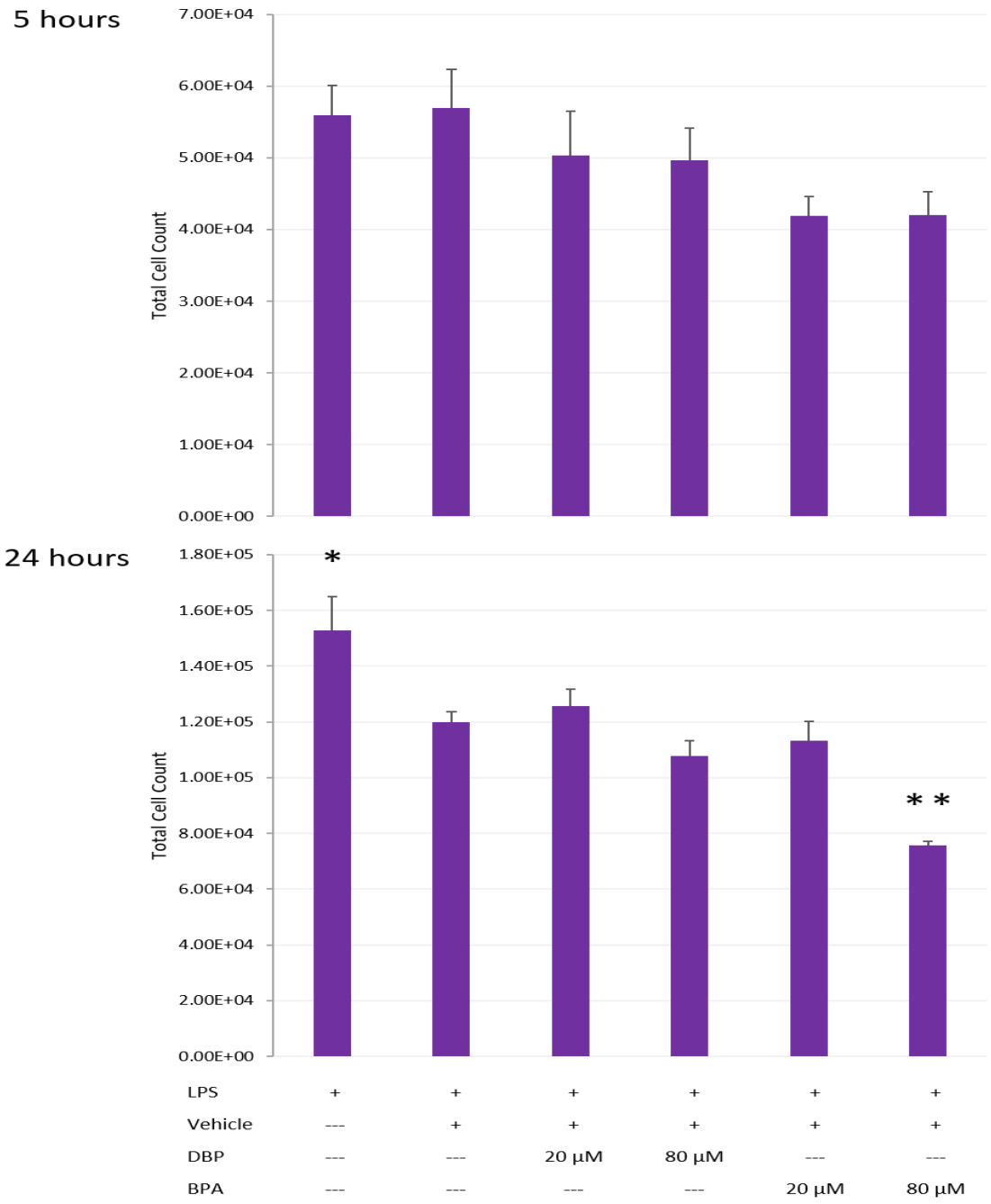

Figure 5. Treatment with BPA Decreases Total Cell Count in RAW 264.7 Cells
RAW 264.7 cells were treated with DBP or BPA at the concentrations indicated for either 5 or 24 hours. Total cell count was quantified using the Countess Automated Cell Counter in conjunction with Trypan blue as described in the Methods. The effect of each treatment was assessed at each time using a multivariate ANOVA followed by Dunnett's *post hoc* test. The vehicle control was used as the reference. (---) and (+) represent the absence or presence of the compound indicated. Asterisks represent groups that are significantly different from the vehicle control (*$p<0.01$, **$p<0.001$). The data is shown as mean ± SEM (n=6).

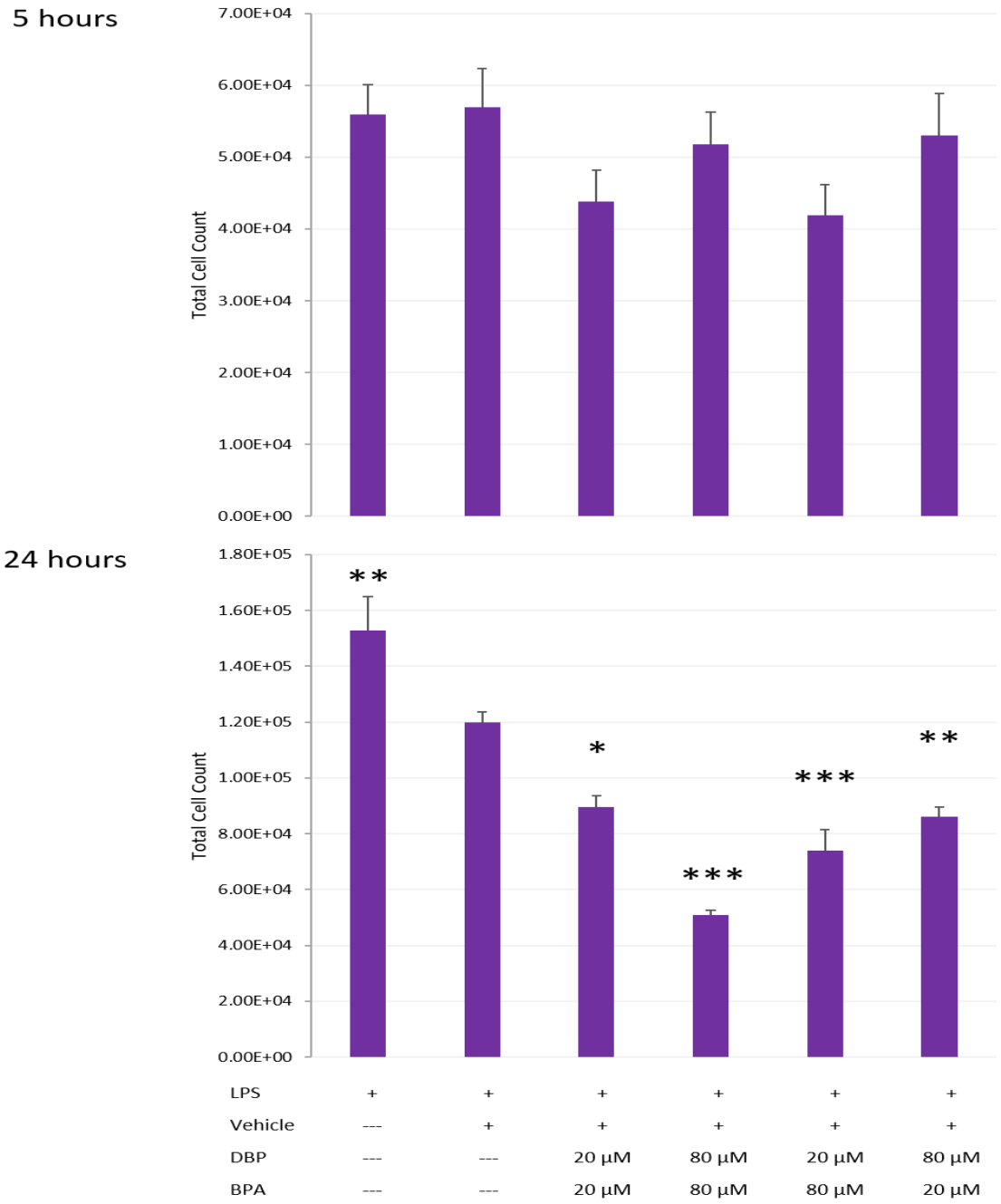

Figure 6. Combination of DBP and BPA Decreases Total Cell Count in RAW 264.7 Cells RAW 264.7 cells were treated with DBP and BPA in combination at the concentrations indicated for either 5 or 24 hours. Total cell count was quantified using the Countess Automated Cell Counter in conjunction with Trypan blue as described in the Methods. The effect of each treatment was assessed at each time using a multivariate ANOVA followed by Dunnett's *post hoc* test. The vehicle control was used as the reference. (---) and (+) represent the absence or presence of the compound indicated. Asterisk(s) represent groups that are significantly different from the vehicle control (*$p<0.05$, **$p<0.01$, ***$p<0.001$). The data is shown as mean ± SEM (n=6).

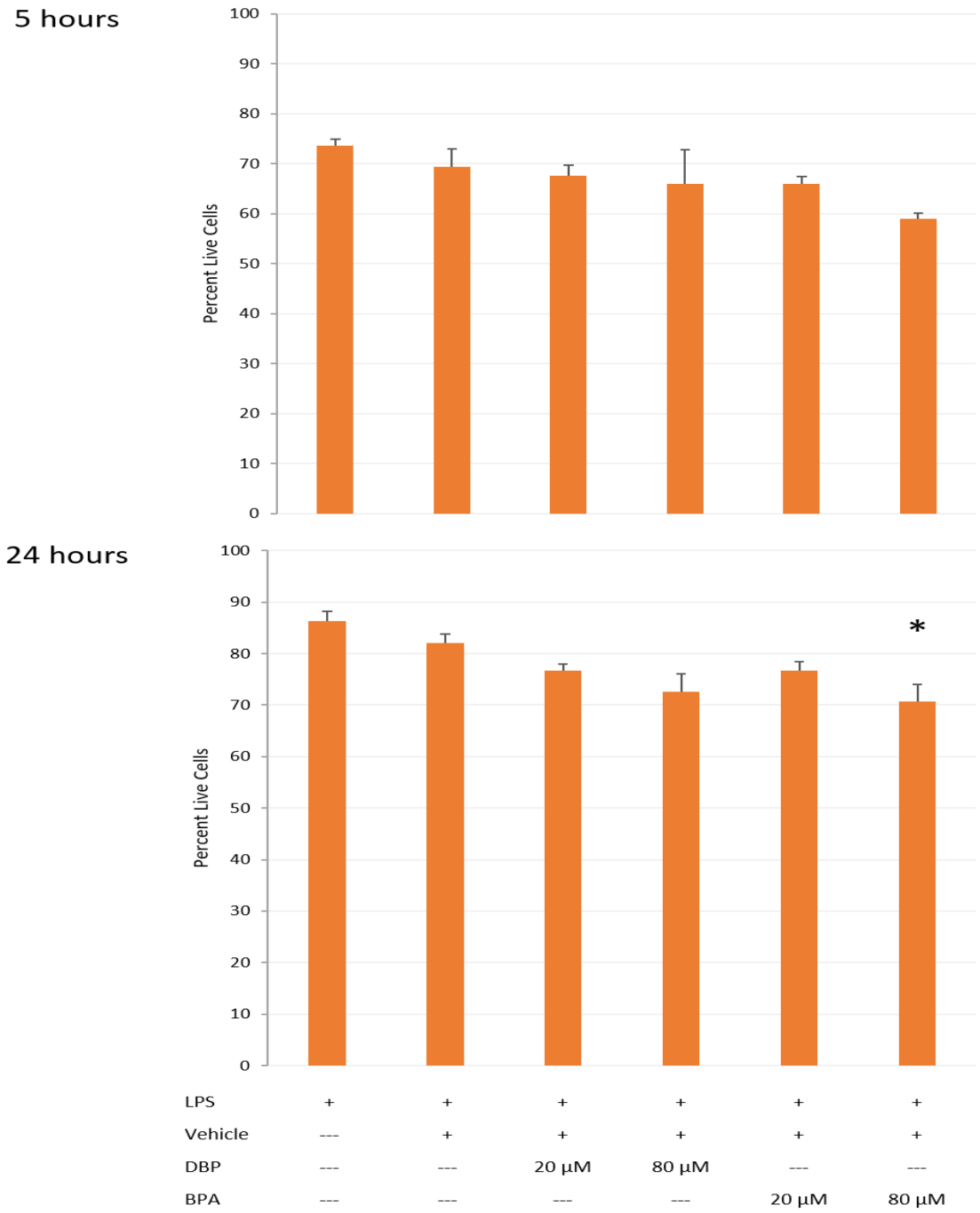

Figure 7. Treatment with BPA Decreases Cell Viability in RAW 264.7 Cells
RAW 264.7 cells were treated with DBP or BPA at the concentrations indicated for either 5 or 24 hours. Cell viability was measured using the Countess Automated Cell Counter in conjunction with Trypan blue as described in the Methods. The effect of each treatment was assessed at each time using a multivariate ANOVA followed by Dunnett's *post hoc* test. The vehicle control was used as the reference. (---) and (+) represent the absence or presence of the compound indicated. * represents groups that are significantly different from the vehicle control (*$p<0.05$) The data is shown as mean ± SEM (n=6).

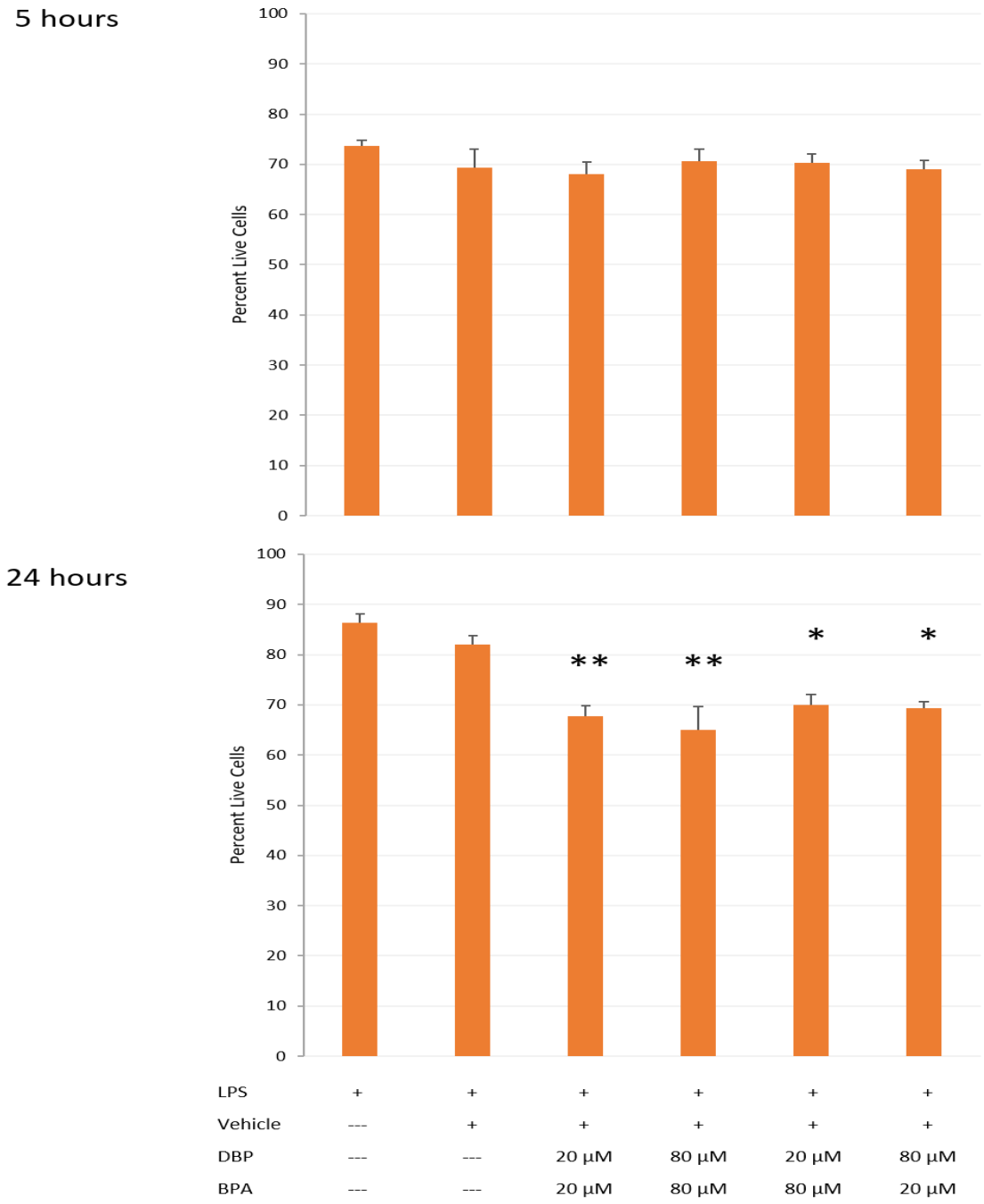

Figure 8. Combination of DBP and BPA Decreases Cell Viability in RAW 264.7 Cells RAW 264.7 cells were treated with DBP and BPA in combination at the concentrations indicated for either 5 or 24 hours. Cell viability was measured using the Countess Automated Cell Counter in conjunction with Trypan blue as described in the Methods. The effect of each treatment was assessed at each time using a multivariate ANOVA followed by Dunnett's *post hoc* test. The vehicle control was used as the reference. (---) and (+) represent the absence or presence of the compound indicated. Asterisk(s) represent groups that are significantly different from the vehicle control (*$p<0.05$, **$p<0.01$). The data is shown as mean ± SEM (n=6).

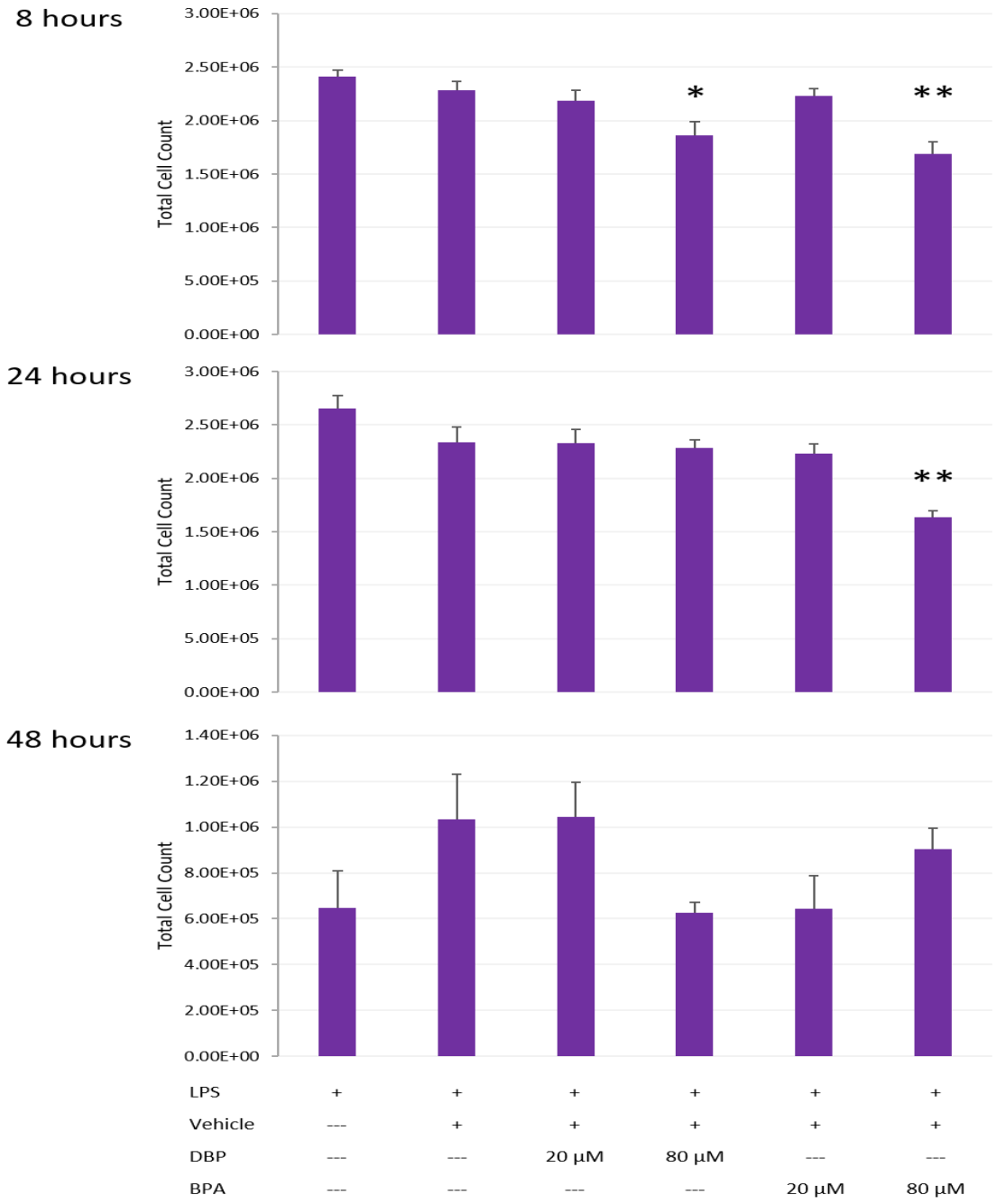

Figure 9. Treatment with DBP or BPA Decreases Total Cell Count after LPS Exposure RAW 264.7 cells were treated with DBP or BPA at the concentrations indicated for 24 hours and then exposed to LPS (1 µg/mL). After 8, 24, and 48 hours, total cell count was quantified using the Countess Automated Cell Counter in conjunction with Trypan blue as described in the Methods. The effect of each treatment was assessed at each time using a multivariate ANOVA followed by Dunnett's *post hoc* test. The vehicle control was used as the reference. (---) and (+) represent the absence or presence of the compound indicated. Asterisk(s) represent groups that are significantly different from the vehicle control (*$p<0.05$, **$p<0.001$). The data is shown as mean ± SEM (n=15).

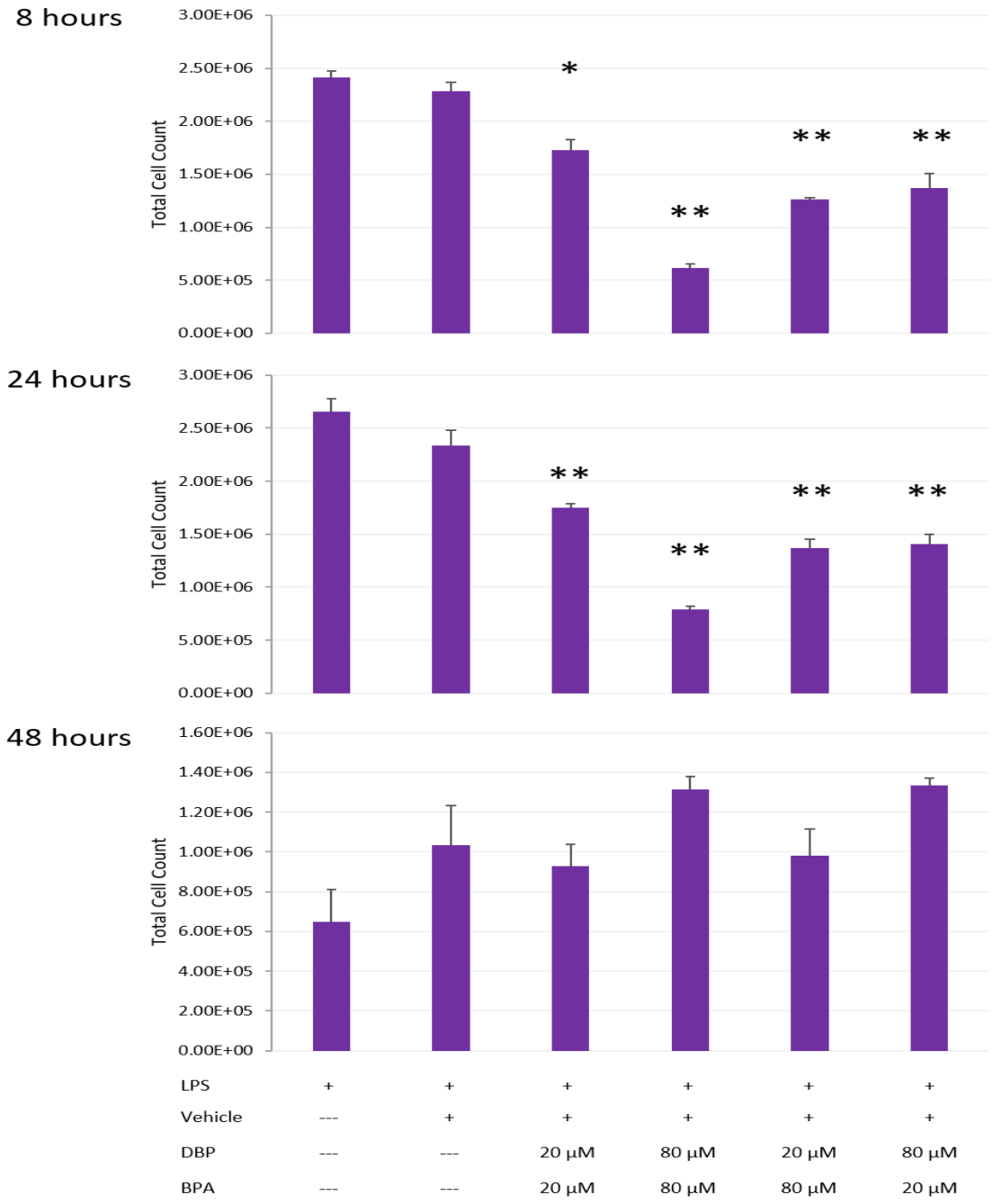

Figure 10. Combination of DBP and BPA Decreases Total Cell Count after LPS Exposure. RAW 264.7 cells were treated with DBP and BPA in combination at the concentrations indicated for 24 hours and then exposed to LPS (1 µg/mL). After 8, 24, and 48 hours, total cell count was quantified using the Countess Automated Cell Counter in conjunction with Trypan blue as described in the Methods. The effect of each treatment was assessed at each time using a multivariate ANOVA followed by Dunnett's *post hoc* test. The vehicle control was used as the reference. (---) and (+) represent the absence or presence of the compound indicated. Asterisk(s) represent groups that are significantly different from the vehicle control (*$p<0.01$, **$p<0.001$). The data is shown as mean ± SEM (n=15).

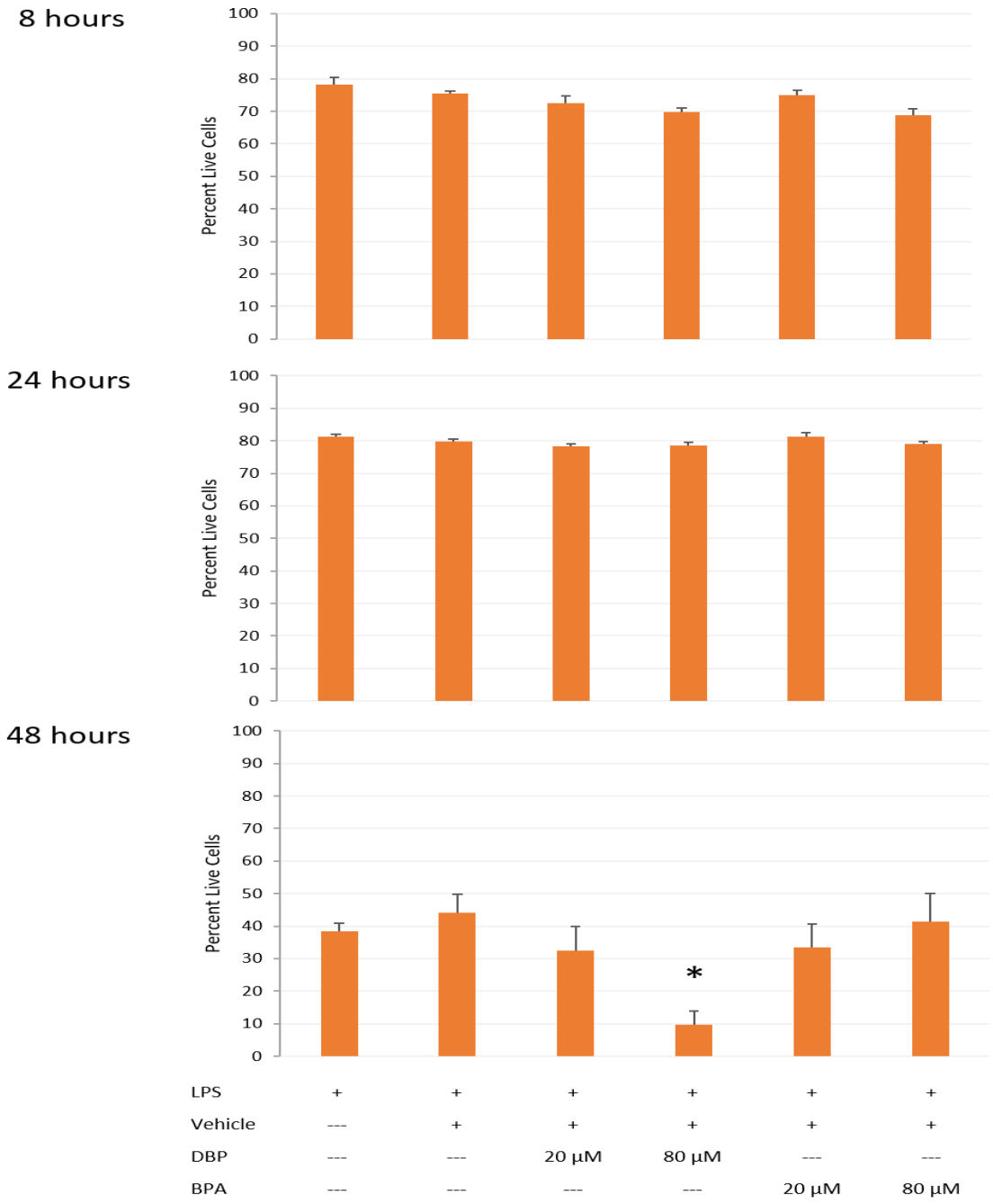

Figure 11. Treatment with DBP Decreases Cell Viability after LPS Exposure
RAW 264.7 cells were treated with DBP or BPA at the concentrations indicated for 24 hours and then exposed to LPS (1 µg/mL). After 8, 24, and 48 hours, cell viability was quantified using the Countess Automated Cell Counter in conjunction with Trypan blue as described in the Methods. The effect of each treatment was assessed at each time using a multivariate ANOVA followed by Dunnett's *post hoc* test. The vehicle control was used as the reference. (---) and (+) represent the absence or presence of the compound indicated. * represents groups that are significantly different from the vehicle control (*$p<0.01$). The data is shown as mean ± SEM (n=15).

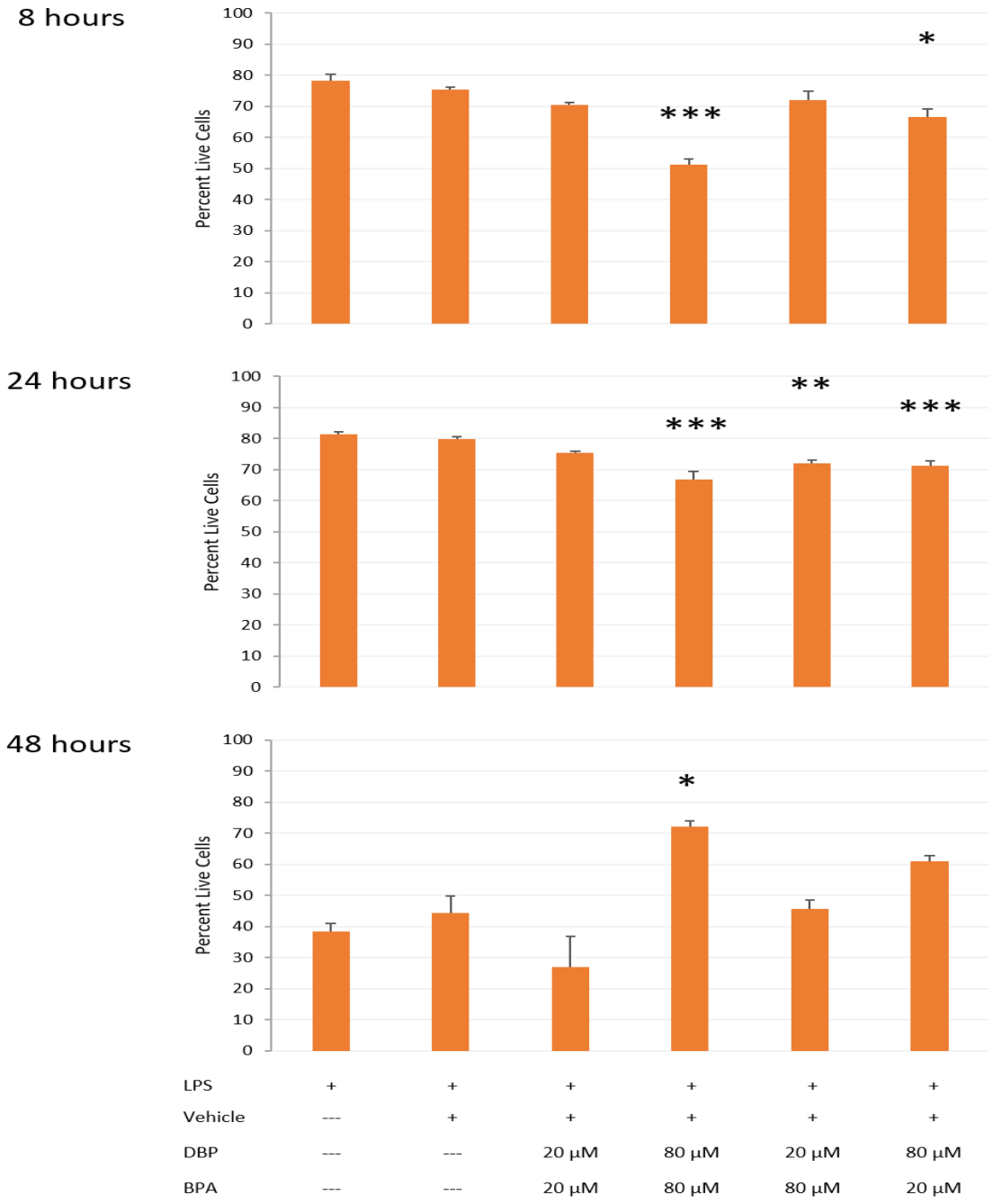

Figure 12. Combined Effect of DBP and BPA on Cell Viability after LPS Exposure
RAW 264.7 cells were treated with DBP and BPA in combination at the concentrations indicated for 24 hours and then exposed to LPS (1 µg/mL). After 8, 24, and 48 hours, cell viability was quantified using the Countess Automated Cell Counter in conjunction with Trypan blue as described in the Methods. The effect of each treatment was assessed at each time using a multivariate ANOVA followed by Dunnett's *post hoc* test. The vehicle control was used as the reference. (---) and (+) represent the absence or presence of the compound indicated. Asterisk(s) represent groups that are significantly different from the vehicle control (*p<0.05, **p<0.01, ***p<0.001). The data is shown as mean ± SEM (n=15).

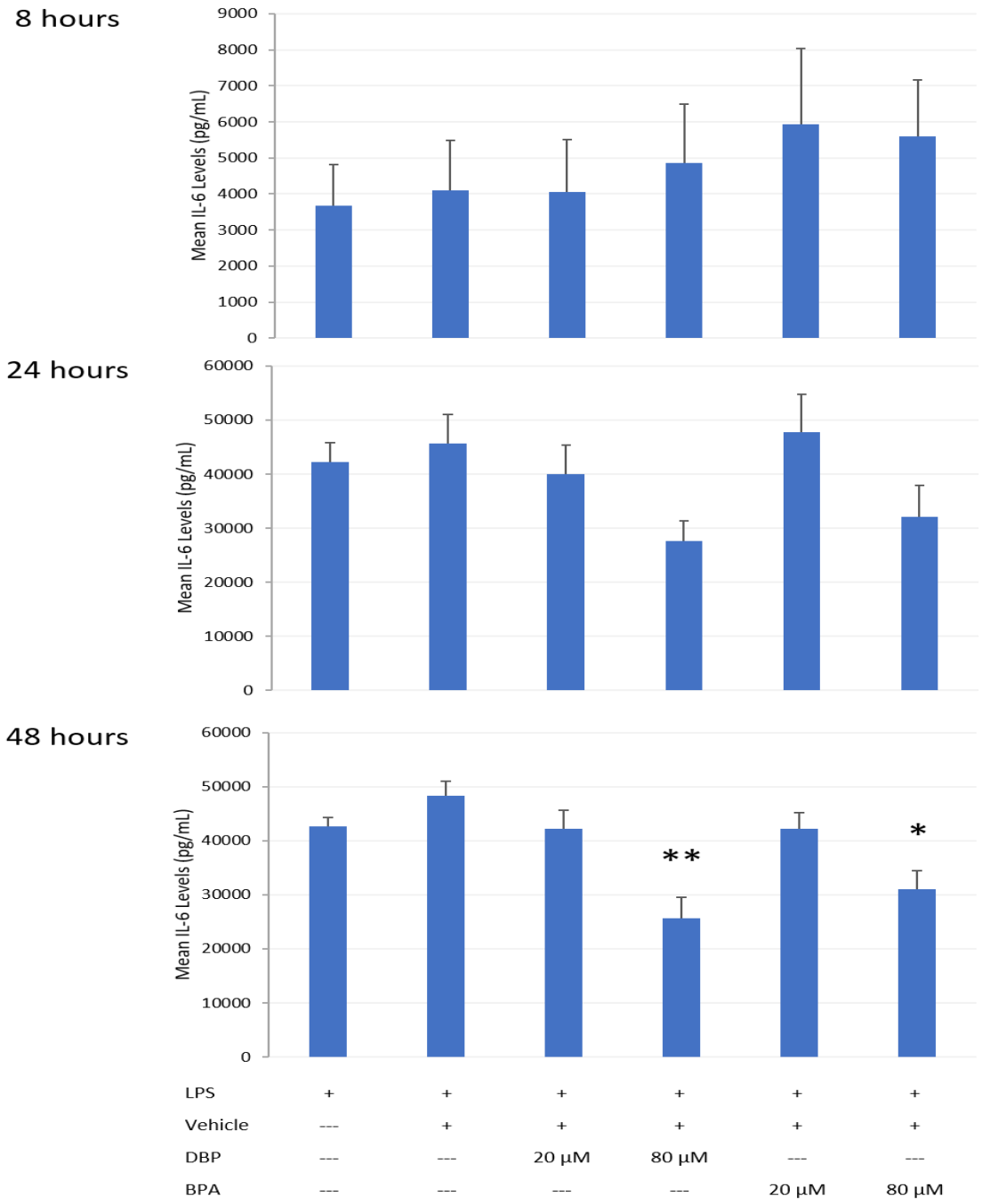

Figure 13. Treatment with DBP or BPA Decreases IL-6 Level after LPS Exposure
RAW 264.7 cells were treated with DBP or BPA at the concentrations indicated for 24 hours and then exposed to LPS (1 µg/mL). After 8, 24, and 48 hours, the media was collected to quantify the level of IL-6 using EIA. The effect of each treatment was assessed at each time using a multivariate ANOVA followed by Dunnett's *post hoc* test. The vehicle control was used as the reference. (---) and (+) represent the absence or presence of the compound indicated. Asterisk(s) represent groups that are significantly different from the vehicle control (*$p<0.01$, **$p<.001$). The data is shown as mean ± SEM (n=15).

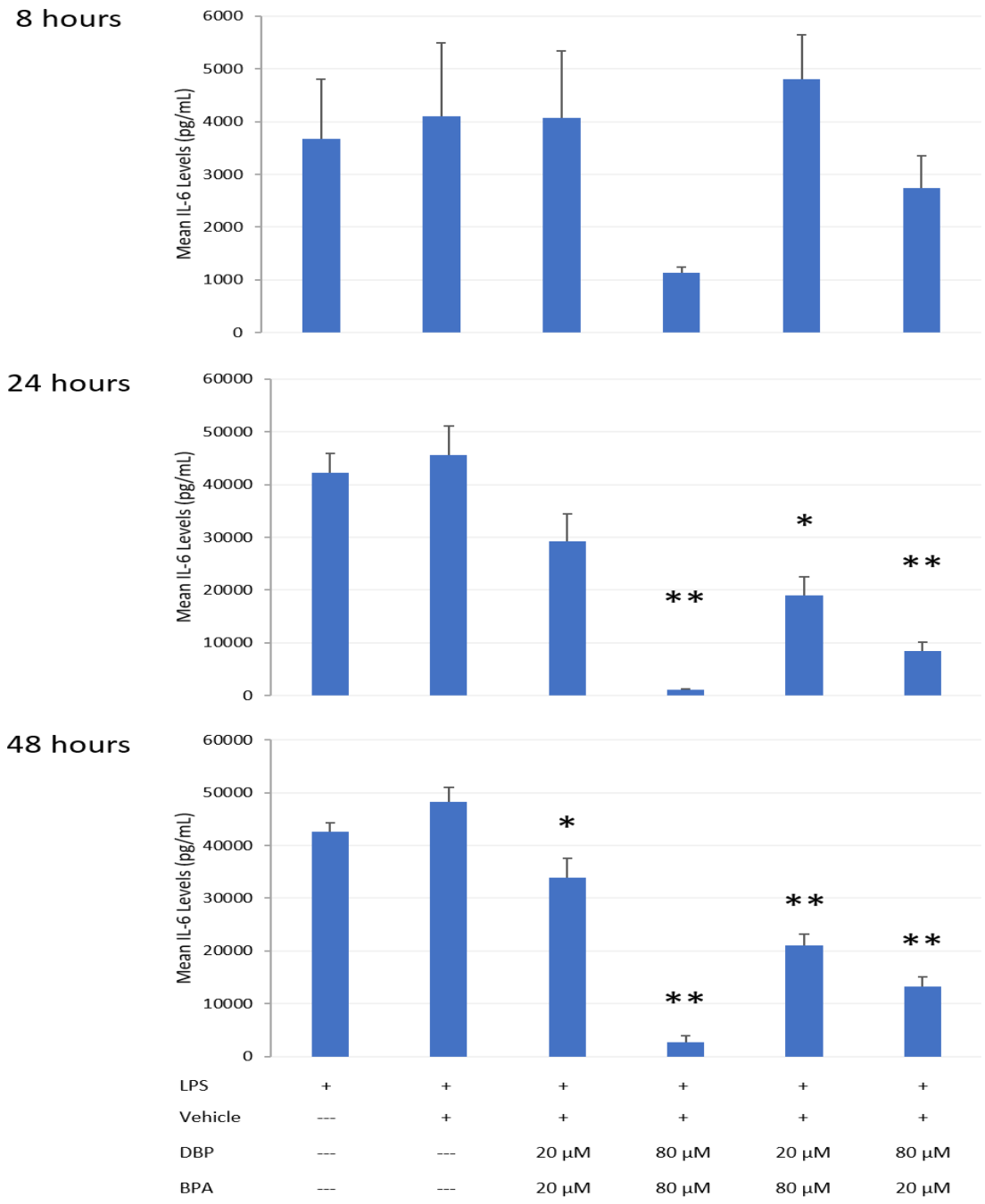

Figure 14. Combination of DBP and BPA Decreases IL-6 Level after LPS Exposure
RAW 264.7 cells were treated with DBP and BPA in combination at the concentrations indicated for 24 hours and then exposed to LPS (1 µg/mL). After 8, 24, and 48 hours, the media was collected to quantify the level of IL-6 using EIA. The effect of each treatment was assessed at each time using a multivariate ANOVA followed by Dunnett's *post hoc* test. The vehicle control was used as the reference. (---) and (+) represent the absence or presence of the compound indicated. Asterisk(s) represent groups that are significantly different from the vehicle control (*$p<0.01$, **$p<0.001$). The data is shown as mean ± SEM (n=15).

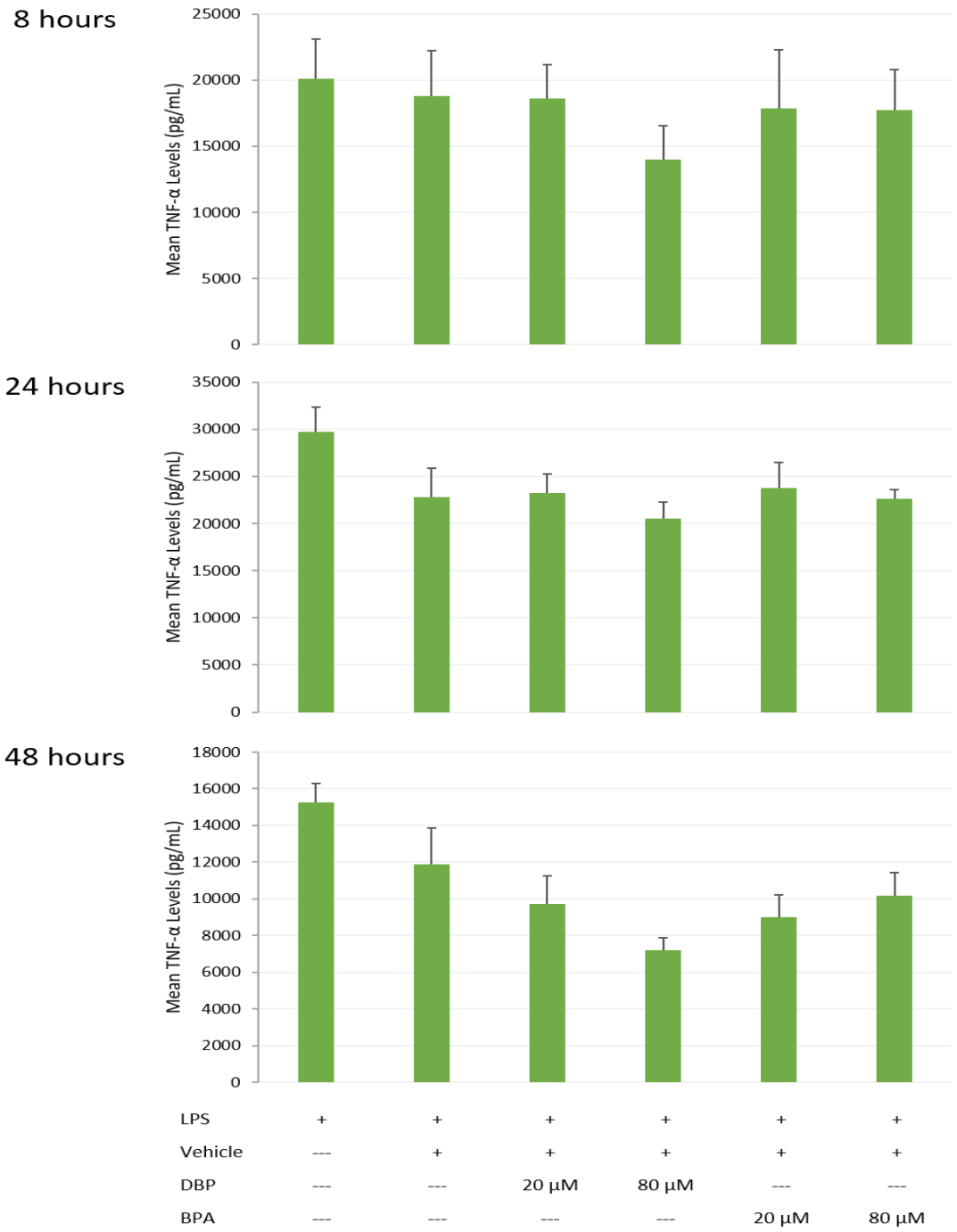

Figure 15. Treatment with DBP or BPA Alone does not Alter TNF-α Level after LPS Exposure. RAW 264.7 cells were treated with DBP or BPA at the concentrations indicated for 24 hours and then exposed to LPS (1 µg/mL). After 8, 24, and 48 hours, the media was collected to quantify the level of TNF-α using EIA. The effect of each treatment was assessed at each time using a multivariate ANOVA followed by Dunnett's *post hoc* test. The vehicle control was used as the reference. (---) and (+) represent the absence or presence of the compound indicated. The data is shown as mean ± SEM (n=15).

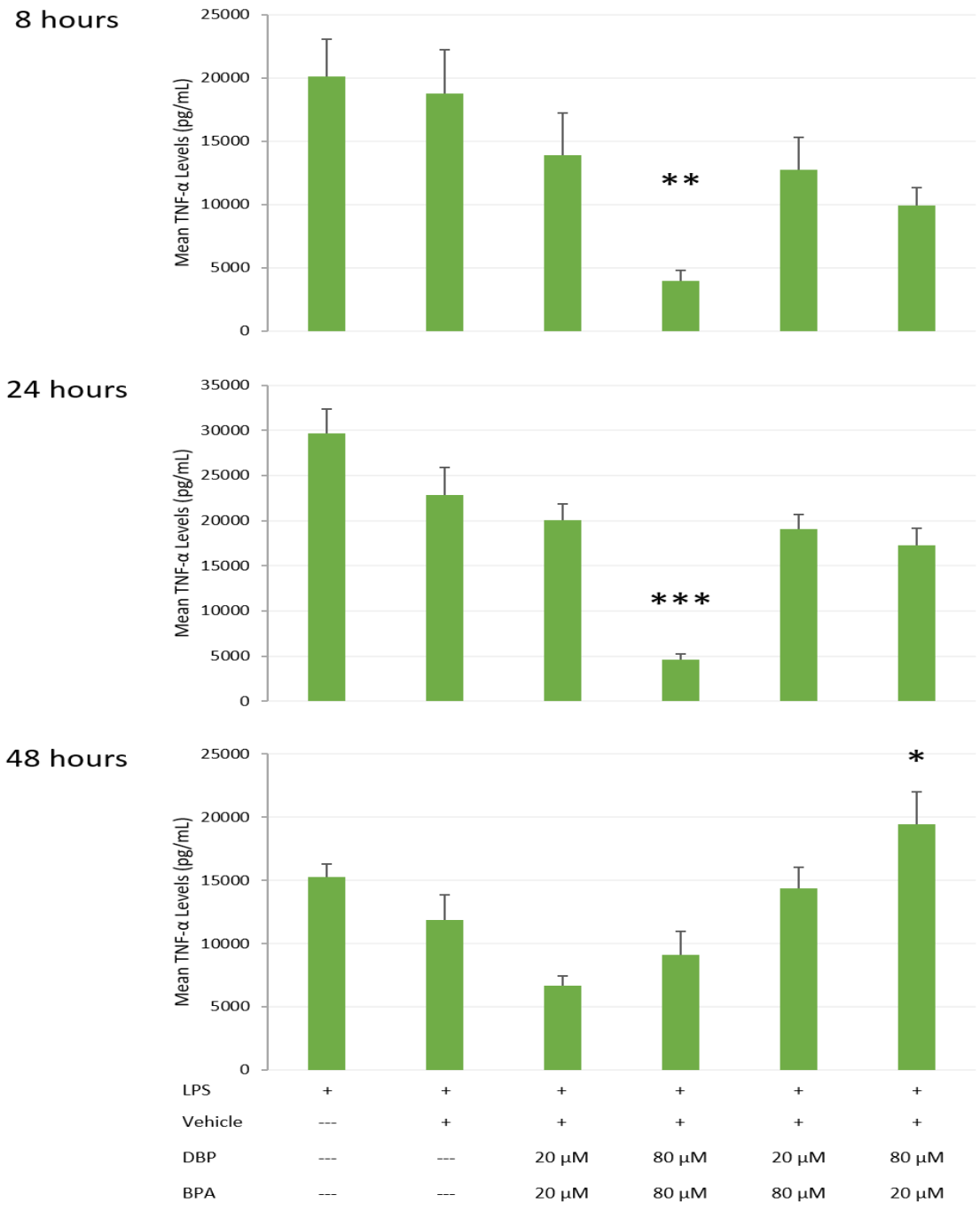

Figure 16. Combined Effect of DBP and BPA on TNF-α Level after LPS Exposure
RAW 264.7 cells were treated with DBP and BPA in combination at the concentrations indicated for 24 hours and then exposed to LPS (1 µg/mL). After 8, 24, and 48 hours, the media was collected to quantify the level of TNF-α using EIA. The effect of each treatment was assessed at each time using a multivariate ANOVA followed by Dunnett's *post hoc* test. The vehicle control was used as the reference. (---) and (+) represent the absence or presence of the compound indicated. Asterisk(s) represent groups that are significantly different from the vehicle control (*$p<0.05$, **$p<0.01$, ***$p<0.001$). The data is shown as mean ± SEM (n=15).

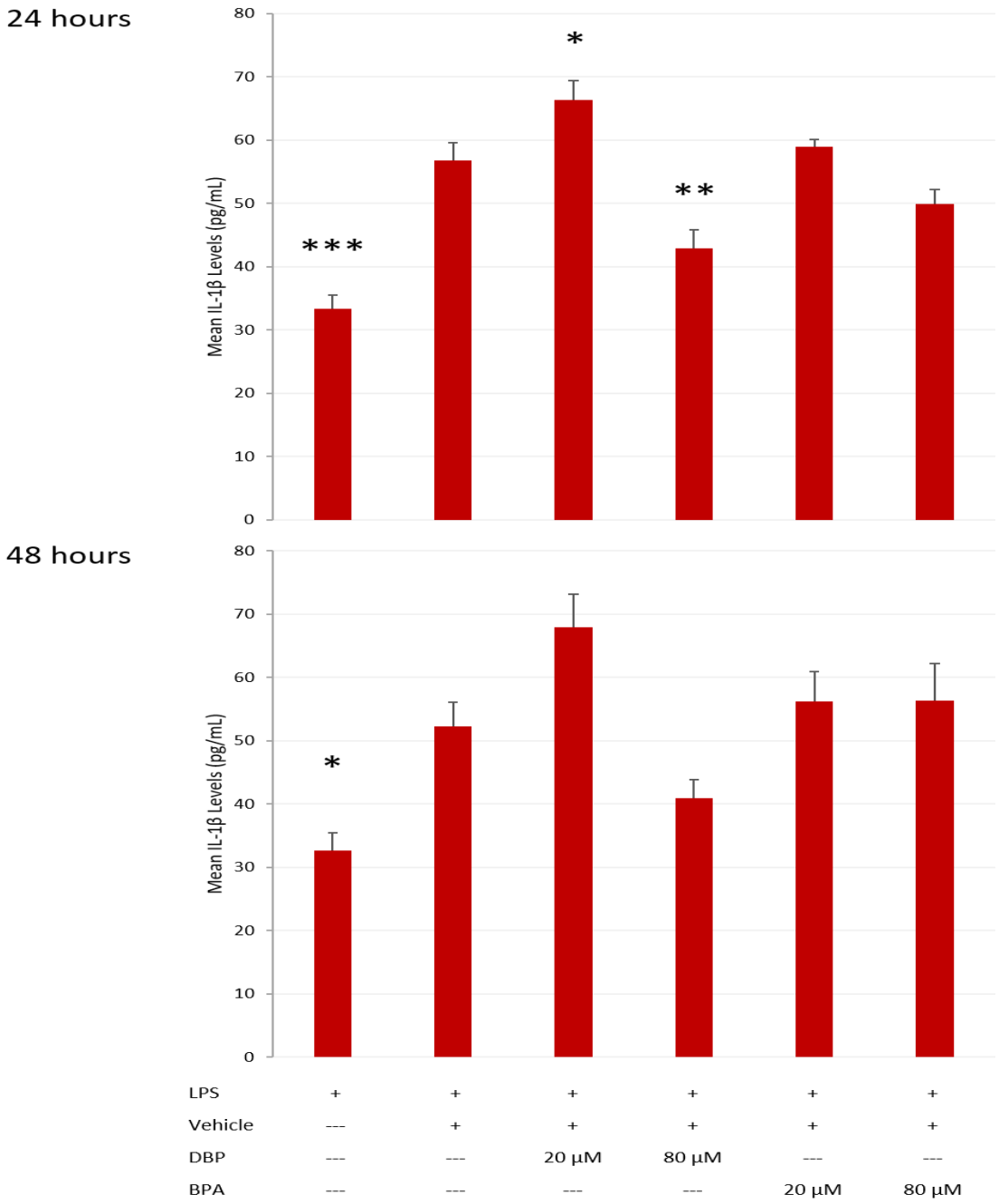

Figure 17. Effect of DBP/BPA on IL-1β Level after LPS Exposure
RAW 264.7 cells were treated with DBP or BPA at the concentrations indicated for 24 hours and then exposed to LPS (1 µg/mL). After 24 and 48 hours, the media was collected to quantify the level of IL-1β using EIA. The effect of each treatment was assessed at each time using a multivariate ANOVA followed by Dunnett's post hoc test. The vehicle control was used as the reference. (---) and (+) represent the absence or presence of the compound indicated. Asterisk(s) represent groups that are significantly different from the vehicle control (*p<0.05, **p<0.01, ***p<0.001). The data is shown as mean ± SEM (n=10).

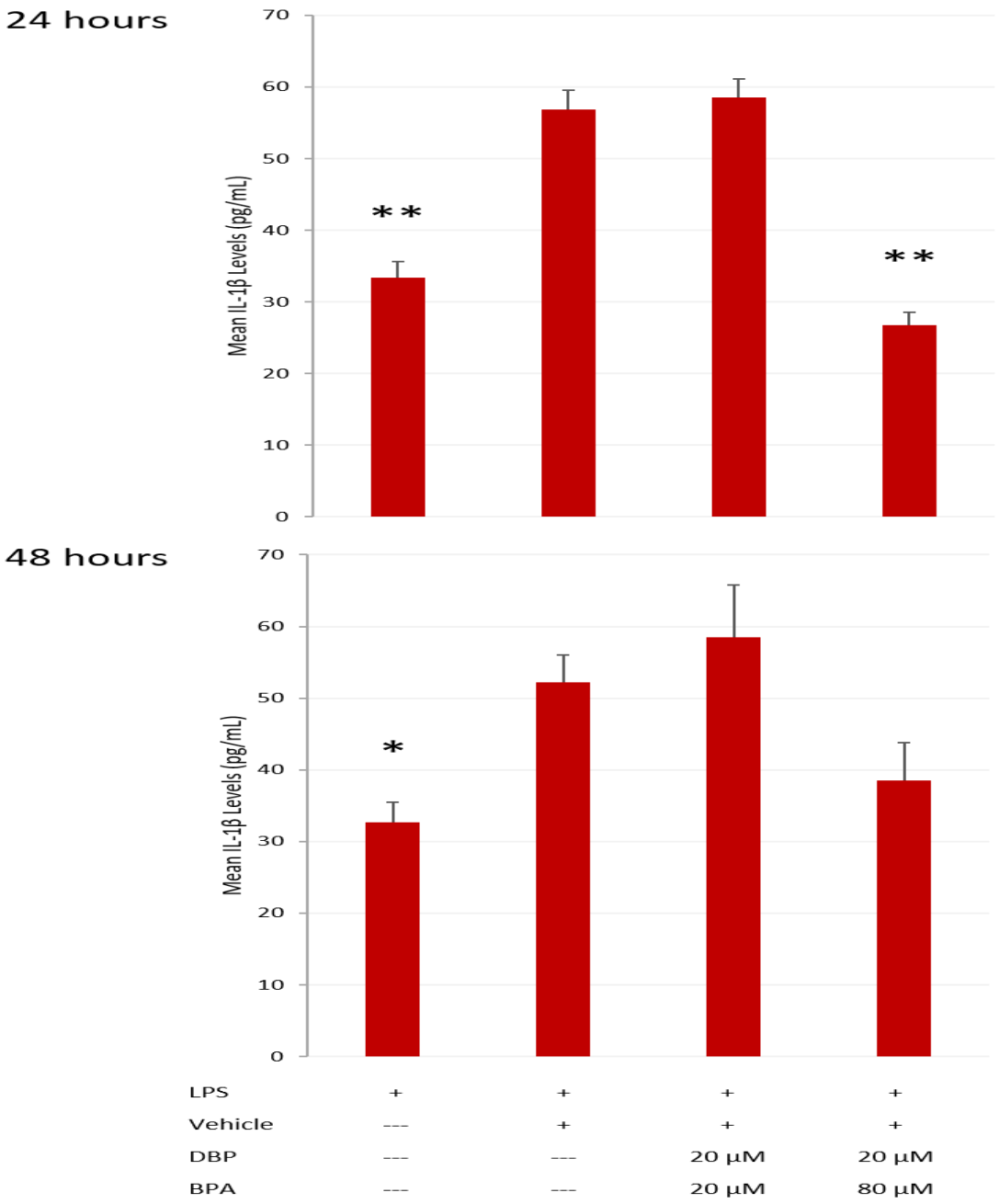

Figure 18. Combined Effect of DBP and BPA on IL-1β Level after LPS Exposure
RAW 264.7 cells were treated with DBP and BPA in combination at the concentrations indicated for 24 hours and then exposed to LPS (1 µg/mL). After 24 and 48 hours, the media was collected to quantify the level of IL-1β using EIA. The effect of each treatment was assessed at each time using a multivariate ANOVA followed by Dunnett's post hoc test. The vehicle control was used as the reference. (---) and (+) represent the absence or presence of the compound indicated. Asterisk(s) represent groups that are significantly different from the vehicle control (*$p<0.05$, **$p<0.001$). The data is shown as mean ± SEM (n=10).

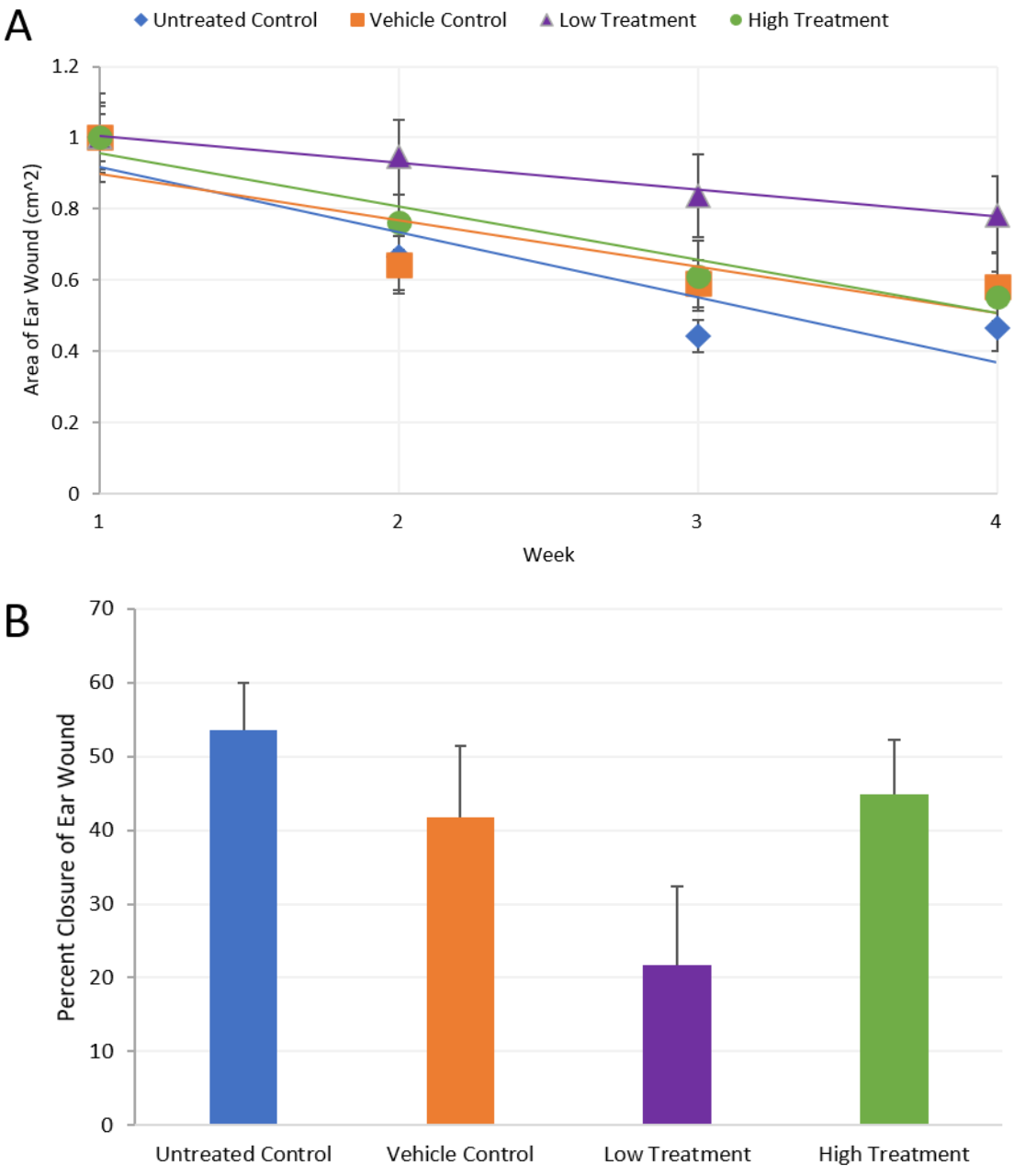

Figure 19. Treatment with DBP does not Alter the Rate of Wound Healing or Overall Wound Closure. C57BL/6J mice were given 2 mm hole punches in their right ears, and the area of the wound was monitored for 28 days, while the mice were given daily injections of different concentrations of DBP (untreated control – no injections; vehicle control – 50 µl of vegetable oil; low treatment – 100 mg DBP/kg body mass; high treatment – 500 mg DBP/kg body mass). The area of each wound was quantified using ImageJ as described in the Methods. (A) shows how the normalized mean wound areas changed over the four weeks (week 1 means 7 days after the initial hole punch). Since the ear wounds were not all initially the same size, percent closure (B) was also calculated. The data is shown as mean ± SEM (n=44).

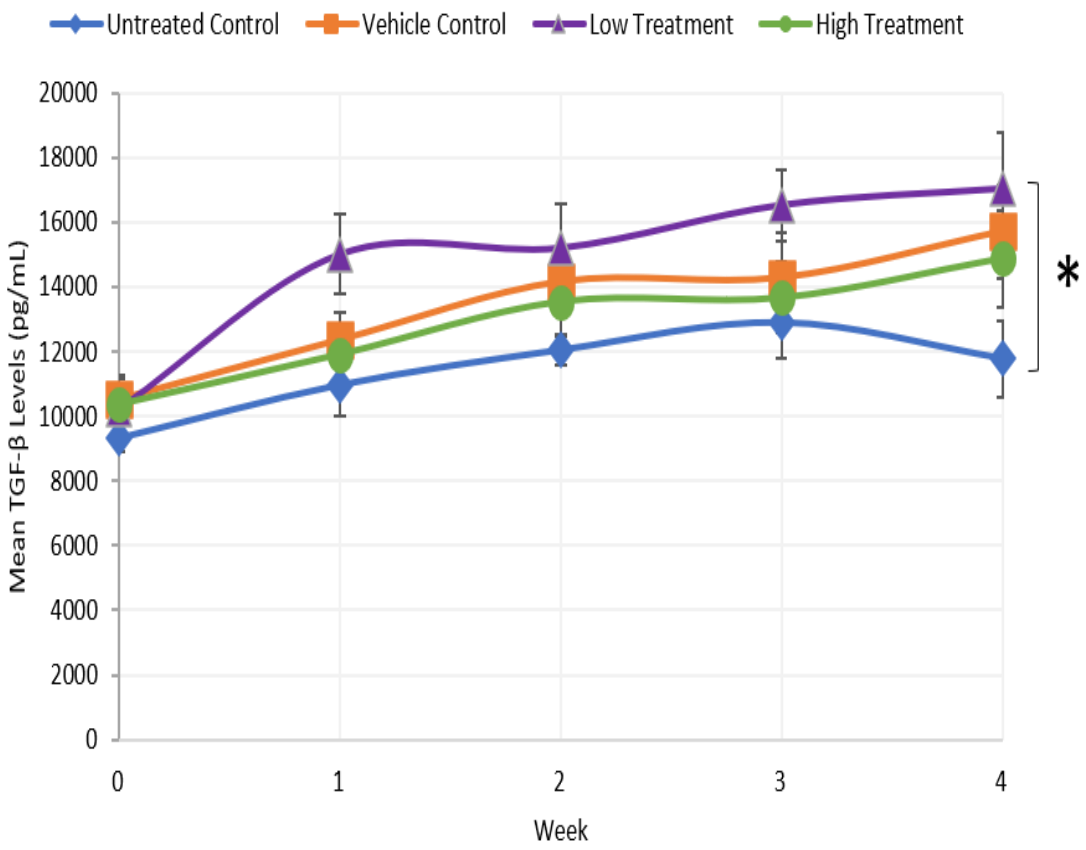

Figure 20. Treatment with DBP Increases Systemic TGF-β Level
C57BL/6J mice were given 2 mm hole punches in their right ears, and the area of the wound was monitored for 28 days, while the mice were given daily injections of different concentrations of DBP (untreated control – no injections; vehicle control – 50 µl of vegetable oil; low treatment – 100 mg DBP/kg body mass; high treatment – 500 mg DBP/kg body mass). Weekly blood samples (~100 µl) were also collected to quantify circulating levels of TGF-β via EIA. * represents significant difference in the overall trend between the untreated control group and the low treatment group. The data is shown as mean ± SEM (n=53).

CHAPTER IV

Discussion

There are at least 1,000 man-made chemicals that have known endocrine-disrupting capabilities (https://www.hormone.org/your-health-and-hormones/endocrine-disrupting-chemicals-edcs), and because of their ubiquitous nature, it is not unreasonable to assume that mixtures of two or more of these chemicals are constantly present within individuals. In fact, in a study conducted in France in 2011, it was found that a variety of contaminants, including bisphenols, phthalates, pesticides, and dioxins, were present simultaneously at quantifiable levels in almost 100% of the pregnant women that were tested (Dereumeaux et al. 2016).

Typically, most individual EDCs are not present at high enough concentrations to affect physiological function on their own, but there are examples from the literature indicating that when combined with other EDCs at low doses they can produce adverse effects. For example, Mu and LeBlanc (2004) found that *Daphnia magna* embryos exposed to fenarimol (a known EDC that disrupts steroid synthesis) in conjunction with testosterone exhibited differential toxicity then either compound alone. In addition, Rajapakse et al. (2002) documented that 11 different xenoestrogens, which included BPA, only had an appreciable effect on 17-β estradiol-induced activation of the ERα receptor when in combination; none had an effect alone.

Effects of DBP and BPA on Total Cell Count

Cellular proliferation is a process that can be modulated by a number of different factors. Briefly, there are four main phases of the cell cycle: G_1, S, G_2, and M. The first

three phases, collectively called interphase, involve cell growth, DNA replication, and damage assessment to ensure there is no DNA damage. M phase involves mitosis (nuclear division) and cytokinesis (cytoplasmic division), resulting in two daughter cells that are identical. The passage from G_1 into the S phase is termed the "restriction point" because at this point the cell is committed to completing cell division.

Progression through these four phases is controlled by many different proteins, mainly cyclins and cyclin-dependent kinases (CDKs). CDKs require cyclins to become activated, and once activated, can phosphorylate different components of the cell cycle. Different combinations of cyclins and CDKs control different phases of the cell cycle. For example, passage through the restriction point is propagated by cyclin E-CDK2 and cyclin D-CDK4/6 (Duronio and Xiong 2013). There is evidence suggesting plasticizer treatment may influence this process (Craig et al. 2013).

In macrophages, the most important pathways governing proliferation are the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway (PI3K/Akt) and the mitogen-activated protein kinase/extracellular signal regulated kinase pathway (MAPK/ERK). Each pathway can be initiated via the cytokine macrophage-colony stimulating factor (M-CSF) when it binds to the colony stimulating factor 1 receptor (CSF-1R). This causes a downstream signaling cascade that eventually leads to the activation of Akt (PI3K/Akt pathway). While Akt activity can regulate many downstream effector proteins, its inactivation of glycogen synthase kinase 3 (GSK3) leads to an upregulation of genes required for cell proliferation, such as the cyclin D1 gene (Zhang et al. 2014). Akt also suppresses the expression of certain CDK inhibitor proteins (CKIs), such as p27, to further promote cell proliferation (Los et al. 2009). Binding of M-CSF to CSF-1R can

also cause a downstream signaling cascade that will lead to activation of ERK, which activates many downstream targets involved in cell proliferation (Lloberas et al. 2016).

Signaling pathways in a cell are usually interdependent and can be activated by many different types of receptors in response to a plethora of stimuli. For example, both the PI3K/Akt and MAPK/ERK pathways can be regulated by the estrogen receptor (ER) signaling pathway. Specifically, when different estrogens, such as 17β-estradiol, bind to either ERα or Erβ, it results in the transcription of genes that regulate the expression of both Ras and PI3K. Ras is a family of monomeric guanosine nucleotide-binding proteins (G proteins) that can regulate cellular signaling based on whether they are bound to guanosine diphosphate (GDP) or guanosine triphosphate (GTP) (Molina and Adjei 2006). Ras and PI3K are essential components of the MAPK/ERK and PI3K/Akt pathways, respectively (Gil 2014; Hong and Choi 2018). ERs are expressed by many different immune cells, such as monocytes, dendritic cells, and macrophages, although which receptor is expressed more, ERα or ERβ, depends on cell type. For example, expression of ERβ was found to be higher in monocytes, while macrophages have a higher expression level of ERα (Cunningham and Gilkeson 2010).

Previous studies have documented the modulation of the PI3K/Akt and MAPK/ERK pathways by both DBP and BPA. For example, DBP inhibits the expression of the cyclin D2 gene in mouse ovarian antral follicles to inhibit follicular growth (Craig et al. 2013). On the other hand, DBP increased proliferation of human breast cancer cells (MCF-7) through upregulation of the PI3K/Akt pathway (Chen and Chien 2014). A similar effect was seen in TM4 cells (a mouse sertoli cell line) *in vitro* via the upregulation of the MAPK/ERK pathway (Ma et al. 2020).

Meanwhile, BPA increases ERK activation in JEG-3 cells (a human placental cell line) (Chu et al. 2017) and Zhao et al. (2014) found that BPA increased the growth of primordial follicles by upregulation of the PI3K/Akt pathway. Finally, both DBP and BPA are classified as xenoestrogens as a consequence of their ability to bind to ERs. In the case of BPA, it is able to bind to both ERα and ERβ to act as either an agonist or antagonist dependent on cell type and anatomical location (Acconcia et al. 2015). However, DBP has very low affinity for ERβ but can act as an agonist of ERα-mediated signaling (Wakui et al. 2013). Hence, it is plausible that cellular proliferation of ER-expressing cells could be affected by exposure to BPA or DBP.

Since previous studies have found that these plasticizers can either upregulate or downregulate cell proliferation, we did expect to see a difference in total cell count. However, we were not sure whether the total number of cells would increase or decrease, because this effect has been largely dependent on cell type. We found that BPA resulted in a reduction in the total cell count of RAW 264.7 cells in the absence of an LPS exposure (i.e. the preliminary experiment) (Figure 5), and that this effect was even more prominent when BPA was used in conjunction with DBP (Figure 6). However, when the cells were exposed to LPS after plasticizer treatment, both DBP and BPA resulted in a decrease in the total number of cells (Figure 9), and an even more significant decrease in cell count was observed when they were delivered together (Figure 10). An important caveat is that that cell counts were taken at immediately after 5 or 24 hours of plasticizer exposure in the preliminary experiment (Methods, Figure 3), while the LPS treated cells went up to an additional 48 hours after exposure prior to cell collection (Methods, Figure 4).

Nevertheless, we speculate that this observed decrease in cell count is due to these plasticizing agents modulating some aspect of the cell proliferation process in this cell line. However, more direct measures of cell proliferation must be employed to determine if this is true. While the assessment of cell counts can be used as a proxy for cell proliferation, it is not a direct measure of cell proliferation. How these compounds are influencing cell proliferation should also be determined. It is possible that DBP and BPA are downregulating some aspect of the PI3K/Akt and/or MAPK/ERK pathways; either by interfering directly with some intermediate component or by altering their ER-mediated modulation. Additionally, the expression of proteins required for cell cycle progression, such as cyclin D, that are regulated by these pathways may be reduced. Further experiments that assess ER occupancy by DBP/BPA and the level of proteins involved in the PI3K/Akt and MAPK/ERK pathways should be assessed to elucidate underlying mechanisms.

Effects of DBP and BPA on LPS-induced Cytokine Level

Cytokines are vital mediators of inflammation and their production and secretion is regulated by many different signaling pathways. In response to LPS, TLR4-mediated signaling leads to the activation of NF-κB (Figure 1) resulting in upregulation of pro-inflammatory cytokines, mainly IL-6, TNF-α, and IL-1β, and prior studies have suggested that plasticizer treatment can affect this process. For example, a pilot study conducted previously in our lab (Davis and Harper, unpublished) indicated that DBP decreased IL-6 level induced by LPS in primary mouse fibroblasts in a dose-dependent manner, although BPA had no effect. In addition, Li et al. (2013) demonstrated that

peritoneal macrophages from C57BL/6 mice treated with DBP exhibited a decrease in LPS-induced production of IL-6, TNF-α, and IL-1β. Neither of these studies examined the effect of these compounds in conjunction, however.

Here we found that exposing RAW macrophages to the high dose (80 μM) of either DBP or BPA decreased IL-6 level (Figure 13), and the presence of 80 μM of DBP alone (Figure 13), or in combination with either dose of BPA (Figure 14), resulted in the greatest decline. Not only did the compounds individually decrease IL-6 level, but the effect was also more pronounced when the two were combined; therefore, for IL-6, we reject our first two null hypotheses (Null hypotheses #1 & #2; Introduction, pg. 20) We found that neither compound individually affected TNF-α level (Figure 15), but an effect was seen when the two compounds were combined. Specifically, a decrease in TNF-α level was seen in the presence of 80 μM of DBP in conjunction with 80 μM of BPA (Figure 16). Therefore, for TNF-α, we reject our second null hypothesis (Null hypothesis # 2; Introduction, pg. 20), but we fail to reject our first null hypothesis (Null hypothesis # 1; Introduction, pg. 20).

We can speculate that these plasticizers, alone and especially in combination, are downregulating some aspect of TLR4-mediated signaling such that the release of pro-inflammatory cytokines is reduced. However, it remains to be seen how this occurs. Possibilities include: (1) DBP acts as a TLR4 antagonist and that this effect is enhanced in the presence of BPA to interfere with LPS binding and the induction of downstream signaling events; (2) these compounds are interfering with other TLR4 signaling components downstream of the receptor, such as NF-κB activity. Consistent with this notion, phthalates, including DBP, are classified as peroxisome proliferators which bind

to and activate members of the peroxisome proliferator-activated receptor (PPAR) family. In turn, these receptors act as transcription factors to alter the expression of genes associated with lipid metabolism, as well as inflammation. Importantly, PPARγ suppresses the inflammatory response through its negative regulation of NF-κB (Li et al. 2013). Each of the three subtypes of PPARs (i.e. PPARα, PPARβ/δ, and PPARγ) are weakly activated by DBP (Lapinskas et al. 2005).

Additionally, it is possible that DBP and BPA may alter other signaling components involved in regulating inflammation, independent of TLR4 activation. For example, whole blood exposed to the drug Resiquimod (R-848), a TLR7/8 agonist, resulted in a reduction of IFN-γ level when in the presence of DBP (Maestre-Batlle et al. 2018).

For IL-1β, it is slightly more complicated. We found that the presence of the vehicle (1% ethanol) alone caused a statistically significant increase in IL-1β level even though it had no effect on IL-6 or TNF-α levels (Figures 17 & 18). Additionally, the presence of 20 μM of DBP alone increased the IL-1β level even further, although in the presence of 80 μM of DBP alone, IL-1β was significantly reduced relative to the vehicle control (Figure 17). When cells were exposed to BPA in conjunction with the 80 μM dose of DBP it often resulted in an undetectable level of IL-1β in the media (data not shown).

While TLR4-mediated signaling does cause the secretion of IL-1β, it is in an inactive precursor form (i.e. pro-IL-1β) which requires subsequent cleavage by the caspase-1 enzyme to reach its mature form. However, caspase-1 itself must first be activated by the formation and activation of an inflammasome, mainly the NLRP3

inflammasome. NLRP3 is comprised of several domains, that ultimately induce the activation of caspase-1 and subsequent cleavage of pro-IL-1β. For example, NLRP3 has a pyrin domain (PYD) which interacts with the PYD on apoptosis-associated speck-like protein (ASC), an adaptor protein. In addition to PYD, ASC also contains a caspase recruitment domain (CARD) which interacts with the CARD on caspase-1, thus promoting its activation (Sharif et al. 2019). All of these components are necessary for proper assembly and activation of this inflammasome. Importantly, activation of NF-κB in the presence of specific TLR ligands (including LPS) upregulates the expression of the NLRP3 inflammasome (Kelley et al. 2019). However, previous studies have documented that RAW 264.7 cells do not traditionally express ASC, the adaptor protein that is required for activation of the NLRP3 inflammasome (Pelegrin et al. 2008). Therefore, we can say that the NLRP3 inflammasome is not being activated in this study. However, we were still able to detect IL-1β in the media (at low levels) after LPS exposure, and it remains to be seen how this occurred. The most likely possibility is that IL-1β is not being induced by LPS and the levels that we found were simply background noise. One way to determine if this is true is to use a control group that is not exposed to LPS; if there is no difference in IL-1β level between the group exposed to LPS and the group not exposed to LPS, then the detected IL-1β is simply noise. Exposing RAW 264.7 cells to LPS will still upregulate TL4-mediated production of pro-IL-1β. Therefore, it is possible that the EIA (Thermofisher/Invitrogen) we utilized is detecting the pro-form released by damaged and/or dying cells rather than the mature form which is actively secreted. The expression of pro-IL-1β should directly be assessed to determine if this is the case. This may help to explain why we found detectable levels of IL-1β at 24 and 48 hours, but the

levels were undetectable at the 8-hour timepoint. Another possibility is that an entirely different inflammasome that is not ASC-dependent, such as the NLRC4 inflammasome, is being activated; although there is very little evidence to suggest this is the case, especially since the observed level of IL-1β was so low. Assessing the level of active caspase-1 may help to determine if this is true. Overall, the IL-1β results remain largely inconclusive. We are not able to accept or reject our null hypotheses because we used the wrong cell line to measure active IL-1β. Therefore, we cannot say if DBP and BPA influence this process.

Effect of DBP on Wound Healing and Systemic TGF-β *In Vivo*

Because inflammatory mediators are crucial to the wound healing process, we expected that treatment with DBP would also affect the rate of wound healing and/or the degree of healing in an *in vivo* model. Unexpectedly, we saw no effect at the macroscopic level (Figure 19), and so we fail to reject our final null hypothesis (Null hypothesis #3; Introduction, pg. 20). However, it may be too early to say that DBP is without consequence. In particular, the greatest degree of healing (i.e. wound closure) was only approximately 50% (Figure 19). Although other studies indicated that 28 days was a sufficient period, we failed to see complete healing in any of the groups. Perhaps this was due to our vivarium being a conventional, rather than specific pathogen free (SPF), facility. If the experiment had gone longer, a difference may have been seen. In addition, we did not examine any measures *at the wound site* itself, including TGF-β, platelet-derived growth factor (PDGF), TNF-α, IL-6 or matrix metallopeptidases (MMPs)

levels. Each of these are important mediators of the wound healing response, with MMPs being involved in virtually all stages of wound healing (Agyare et al. 2019).

Instead, we hoped to see a difference using systemic (i.e. serum or circulating) TGF-β as a marker. Similar to MMPs, TGF-β is involved in virtually all stages of the wound healing process, especially fibrosis (Ramirez et al. 2014), and is a member of the TGF-β superfamily comprised of an array of factors involved in the regulation of cellular homeostasis, proliferation, and function. Although it is secreted by many cells, including fibroblasts, dendritic cells, and macrophages, TGF-β synthesis and release is a complicated process. In particular, it is actually produced as a large latent complex that is comprised of TGF-β covalently bonded to proteins latency-associated peptide (LAP) and latent TGF-β binding protein (LTBP), which is usually tethered to the extracellular matrix until TGF-β is needed. This entails the release of the TGF-β molecule via proteolytic cleavage or changes in pH. This free (active) TGF-β then goes on to interact with serine-threonine kinase receptors to initiate the canonical SMAD-dependent signaling pathway in addition to SMAD-independent transducers (Ashcroft 1999; Nüchel 2018).

We found that 100 mg DBP/kg body weight significantly increased serum TGF-β level when compared to the untreated control only (Figure 20). Although this finding is in accordance with a previous study (Zhu et al. 2017) whereby DBP significantly increased the expression of TGF-β, this was *in vitro* in the rat kidney cell line NRK-52E. The higher dose of DBP (500 mg/kg body weight), however, had no significant effect (Figure 20). The reason for this discrepancy is unknown, but it is notable that the specific factor(s) responsible for the regulation of circulating TGF-β level in the absence of overt

tissue damage remains unclear. Moreover, while the presence of tissue damage or certain pathologies is known to result in a marked increase in TGF-β, the response is varied. For example, strenuous physical activity, which damages muscle fibers, can increase serum levels of many different growth factors, including TGF-β (Czarkowska-paczek et al. 2006). Since we did see an increase in TGF-β level across all groups with time (Figure 20), our finding is consistent with the notion that the damage caused by the wounding led to a response.

Of note, the EIA (Thermofisher/Invitrogen) we used to quantify serum TGF-β involved a pretreatment step in which the active form of TGF-β was released from its latent form using hydrochloric acid. Therefore, the assay measured total TGF-β: both free TGF-β that was present even prior to acidification and dissociated latent TGF-β. Future experiments should attempt to quantify both latent TGF-β and free TGF-β individually.

Conclusion and Future Directions

Both DBP and BPA, individually and in combination, had some effect on the number of RAW 264.7 cells present over time, both in the absence and presence of LPS. Cell viability was minimally affected, however. Hence, it is believed that these compounds may alter the cellular proliferation process in this cell line, although the underlying mechanism remains unclear. The first step is to determine if this is true by utilizing more direct measures of proliferation, such as assessment of Ki-67 activity. Then, markers of cell cycle progression, such as cyclin D or p27, as well as the level or activity of other proteins involved in cell cycle regulation can be assessed to determine how the proliferation process is being affected.

We also found that plasticizers can reduce the level of both IL-6 and TNF-α in cultures of RAW 264.7 cells after LPS exposure via an unknown effect. Modulation of TLR4 signaling and the subsequent expression of these cytokines is thought to be a likely candidate, however, it is also possible that cytokine secretion itself is impaired. Examination of TLR4 receptor level, TLR4 occupancy by DBP/BPA, NF-κB translocation or exocytic vesicle formation are all suggested as a follow up to aid in pathway elucidation. The effect of DBP and BPA on LPS-induced IL-1β level remains unknown due to an incorrect model being employed. The study should be repeated using a cell line that is known to express ASC and can, therefore, activate the NLRP3 inflammasome, allowing for proper secretion of active IL-1β.

Finally, while we did not see an effect of DBP on the wound healing process, we did find that the circulating level of TGF-β was significantly increased in the presence of the low dose of DBP used in this experiment. The assessment of local biomarkers at the wound site itself using techniques like immunohistochemistry could provide important insight. Additional wound healing models, such as full thickness skin wounding, or even *in vitro* wound healing assays, are other alternatives.

Overall, these data suggest that exposing cellular mediators of inflammation to the plasticizers dibutyl phthalate or bisphenol A, especially in combination, has significant effects on inflammatory response *in vitro*. To what degree these effects translate *in vivo* remains to be seen, especially in the context of human health and disease.

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RESEARCH EXPERIENCE

Masters Research, Sam Houston State University, January 2018 – December 2020

- Determined the effects of dibutyl phthalate and bisphenol A treatment on level of pro-inflammatory cytokines induced by lipopolysaccharide in RAW 264.7 cell line
- Determined the effects of dibutyl phthalate on wound healing response in C57BL/6J mice
- Techniques utilized during research: Mouse handling/research, cell culture, ELISA, cell viability assays

TEACHING/MENTORING EXPERIENCE

Teaching Assistant, Sam Houston State University, August 2018 – December 2019

- Laboratory instructor for multiple sections of introductory cell biology, with 15+ students per section.
- Demonstrated different introductory cell biology techniques and evaluated students based on performance, write-ups, and quizzes.

Teaching Assistant, Sam Houston State University, January 2018 – May 2018

- Laboratory instructor for multiple sections of contemporary biology, with 15+ students per section.
- Demonstrated different contemporary biology techniques and evaluated students based on performance, quizzes, and exams.

Science Mentor, University of Houston-Downtown, February 2016 – April 2016
Assisted general microbiology students in experiment setups and laboratory write-ups.

Science Mentor, University of Houston-Downtown, October 2015 – November 2015
Assisted students in research write-ups for freshman biology seminar.

HONORS & AWARDS

Recipient, COSET Graduate Achievement Scholarship, Sam Houston State University, January 2020

Recipient, Student Research Proposal Grant, Texas Academy of Science, March 2019

Recipient, COSET Graduate Achievement Scholarship, Sam Houston State University, January 2019

Recipient, COSET Graduate Achievement Scholarship, Sam Houston State University, August 2018