**Senior Thesis Research Proposal:**

# The Effect of Bisphenol A (BPA) Substitutes on CD4+ T Cell Immunity

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## Introduction

The human immune system is complex and involves a variety of different cell types. One immune system cell type that plays an important role in fighting off pathogens, infections, and cancer is the T cell. T cells come in many different forms, including CD4+ T cells, also called helper T cells or effector T cells, which are responsible for recruiting other immune system cells to defend the body against infection.[1] Naive CD4+ T cells differentiate into several effector T cell types, depending on what cytokines they are exposed to. Four major classes of effector T cells are Th1, Th2, Th17, and Tregs, and each of these classes produces its own cytokines and performs its own functions within the immune system (Figure 1).[2,3,4]

Th1 cells produce the cytokines (IFN)-ɣ, (IL)-2, and tumor necrosis factor α, and have antiviral and antibacterial properties.[1,2] Th2 cells produce the cytokines IL-4, IL-5, IL-13, and are involved in defense against extracellular pathogens. Th17 cells play an important role in defense against fungal and bacterial infections, and secrete the cytokines IL-17A, IL-17F, IL-22,[1,2,3] while Tregs are important in the suppression of the immune system, and contribute to the maintenance of bodily homeostasis. For this reason, Tregs have been identified as a key player in autoimmune diseases.[1,2,3] Pathogenic Th1 and Th17 cells are also thought to play a role in autoimmune diseases.[3]

One key factor in the mediation of the immune system in general, and T cells in particular, is the female sex hormone estrogen (17β-estradiol). Estrogen is responsible for regulating the female reproductive system, plays an important role in the process of pregnancy, and is thought to be linked to the development of some autoimmune diseases and cancers.[5,6] Estrogen signaling in the body depends mainly on two types of

nuclear receptors, ERα and ERβ. By binding to these receptors, estrogen is able to trigger signaling pathways and control expression of estrogen-related genes. Estrogen receptors are expressed in most cells of the immune system.[5,7] Estrogen signaling is also involved in the development of cancer, as estrogen plays a key role in cell proliferation and cell cycle progression. Sustained estrogen exposure is a well known risk factor in the development of cancers, including endometrial cancer, prostate cancer, ovary cancer, colon cancer, and lung cancer, but chiefly breast cancer.[8,9] One study found that the estrogen receptor ERα was present in 75% of breast tumors at the time of diagnosis,[10] and therefore antiestrogenic strategies are the leading treatment option for individuals with breast cancer.[9]

Given that estrogen plays such a key role in the body, and sustained exposure is linked to both cancer and autoimmune diseases, the existence and abundance of environmental estrogen mimicking compounds is of particular concern. Estrogen mimics are man-made endocrine disrupting compounds (EDCs) that are found in a variety of everyday products, including plastics, pesticides, medicines, detergents, cosmetics and personal care products.[7] Estrogen mimics are able to bind to estrogen receptors, and therefore act like another source of estrogen in the body. Estrogen mimicking compounds have been linked to many adverse health issues, including cancer, developmental disorder, autoimmune diseases, cardiac diseases, neurological diseases, and obesity.[7,11]

One predominant estrogen mimicking compound is bisphenol A (BPA). BPA is a chemical that is used in the manufacturing of polycarbonate plastics, epoxy resins, and polyester styrene resins.[12,13] The main source of human exposure to BPA is through

diet. BPA has been used in the creation of a variety of food packaging. It can be found in the liners in metal cans for food and beverages and plastic packaging and containers.[8,11,12,14] BPA can be leached from these items into the food and drink that people consume as a result of changes in temperature, pH, and UV light exposure.[8] One study found a positive correlation between eating and drinking canned foods and beverages, and the amount of BPA present in urine samples.[14]

BPA can also be found in toys, medical and dental equipment, electronics, personal care products, and medicines. BPA can be absorbed dermally through contact with thermal paper such as receipts. BPA can also be absorbed through inhalation of contaminated dust.[14,15] Three million tons of BPA are produced each year worldwide, and according to the Food and Drug Administration, more than one billion pounds of that BPA leaks into the environment each year.[8] One study found that more than 90% of people had detectable levels of BPA in their urine samples.[15] During pregnancy, BPA can pass through the placenta to the fetus, and it has been detected in fetal cord blood, amniotic fluid, and breast milk.[13]

BPA exposure plays a role in a number of health concerns. BPA is an estrogen mimicking compound that can bind to both ERα and ERβ estrogen receptors, and has been linked to autoimmune diseases through several different mechanisms related to the immune system (Figure 2).[11] BPA can play a role in shifting the immune system Th1/Th2 profile, which can alter the immune system response to antigens and diseases.[13] One study found that BPA exposure can decrease the number of Treg cells in the innate immune system,[13] and another found that BPA can stimulate cell proliferation, as well as uterine, vaginal, and mammary cell growth and differentiation.[15]

BPA has also been linked to breast cancer. Prenatal exposure to BPA can increase the susceptibility of mammary tissue to tumor promoting factors later in life, while adult exposure can promote the development of estrogen-dependent tumors.[11] It has also been shown that BPA stimulates the migration and invasion of cancer cells, and reduces apoptosis of breast cancer cells, possibly through decreased expression of the tumor suppressor p53.[8]

Understanding of the detrimental health effects of BPA exposure is still relatively new, and BPA is still being produced and used in many industries today. Still, some action has been taken in the last two decades to move away from BPA use as the negative effects become more apparent. In 2008, Canada banned the use of BPA in baby bottles, as did France in 2010 and the European Union in 2011. The US FDA banned the use of BPA in baby bottles and the packaging of infant formula in 2012.[14] As countries and industries are moving away from BPA usage, many replacement compounds have been developed and put into use. Unfortunately, in many cases the safety of these replacement compounds is vague and understudied, as is the role they may play in the human immune system.

There are dozens of BPA substitutes that have been developed in recent years, many of which are structurally similar to BPA. Some of the most common are bisphenol AF (BPAF), bisphenol S (BPS), tetramethyl bisphenol F (TMBPF), 9,9-bis (4-hydroxyphenyl)-fluorene (BHPF), and 1,7-bis(4-Hydroxyphenylthio)-3,5- dioxaheptane (DD-70) (Figure 3). BPS, BHPF, BPAF, and TMBPF are used as replacements in the manufacturing of many plastics and epoxy resins,[14] while DD-70 is mostly used in the manufacturing of thermal papers.[16] As analogues to BPA, each of these compounds

has the potential to act in a similar endocrine-disrupting manner in the body. Previous studies have demonstrated that BPS, one of the most common replacements, has a similar albeit weaker effect on the body as does BPA.[13,14]

BPHF is often used in “BPA-free” bottles. A previous study demonstrated that BPHF can leach from these bottles into water, and that it can have strong anti-estrogenic effects in mice. This is concerning, as antiestrogens are often detrimental to pregnancy.[6] Meanwhile, BPAF has been shown to have an even stronger estrogenic effect than BPA, and caused major developmental defects in zebrafish.[16] There are only a few previous studies that have been done on DD-70 or TMBPF as potential endocrine disrupting compounds. One of those studies found that exposure to DD-70 reduced embryo viability in chickens,[16] while another offered preliminary evidence that TMBPF has little estrogenic activity and may be safer than other BPA alternatives.[17] There do not appear to be any studies on the estrogenic activity of DD-70.

**Specific Aims**

Estrogen is known to play a role in CD4+ T cell differentiation, and is an important hormone in the female reproductive system and for the process of pregnancy. Estrogen also plays a role in the development of several forms of cancer, and may play a role in certain autoimmune diseases. There are numerous compounds that mimic the behavior of estrogen by binding to estrogen receptors. The role of these estrogen mimics in the immune system is an area of active study. One estrogen mimic is BPA, which has been shown to have an effect on the immune system, and may play a role in cancer development and autoimmune diseases (Figure 2). As the detrimental health effects of BPA have begun to emerge, numerous replacement compounds have been

developed, many of which are structurally similar to BPA (Figure 3). However, the effect of many BPA substitute compounds on the immune system, and T cell differentiation in particular, is unknown. The estrogenic or antiestrogenic effects of some BPA substitutes have been investigated by a small number of studies, although others have seemingly not been explored at all. The aim of this study is to begin to address some of these knowledge gaps.

- Aim 1: Do the selected BPA substitutes affect CD4+ T cell differentiation?
- Aim 2: Do the selected BPA substitutes have estrogenic activity in CD4+ T cells, similar to that of BPA itself?

## Materials and Methods

*Cell Stimulation:* Frozen murine CD4+ T cells from C57BL/6 mice will be stimulated for 24-hours with cytokines IL-12, IL-4, IL-6, or TGF-β to induce differentiation of cells to Th1, Th2, Th17, or Treg, respectively, or with media alone for naive CD4+ T cells (Th0). This will be done in triplicate in 96-well plates. The treatments will be with either a control (specific skewing condition alone) or one of the following:

- DMSO (dimethyl sulfoxide, negative control)
- Estrogen (positive control)
- BPA and BPA substitutes (BPS, BHPF, BPAF, TMBPF, DD-70)

*Cell Viability Assay:* T cell death will be determined using the Lactate dehydrogenase (LDH) cytotoxicity assay. Cell death will be measured by the amount of LDH found within the supernatant that is collected. LDH Cytotoxicity Assay Kit (Pierce) will be used according to the manufacturer's instructions.

*Proliferation Assay:* T cell proliferation will be measured by using MTT cell proliferation assay by (Promega) according to the manufacturer's instructions. Spectrophotometry will be used in colorimetric assays to determine the amount of cell proliferation that occurred.

*Cytokine Detection ELISA:* Enzyme-linked immunosorbent assays (ELISA) will be used to detect and quantify cytokine production. Once the cells have been stimulated and incubated, the cell-free supernatants will be analyzed for IFN-γ, IL-4, IL-17, or IL-10 cytokine protein secretion using the ELISA assay (Biolegend), according to manufacturer's instructions.

*RT-PCR:* RT-PCR (reverse transcriptase polymerase chain reaction) will be used to measure the expression of genes that will determine T cell subset differentiation. Using ReliaPrep RNA Cell Miniprep system (Promega), RNA will be isolated from the cells according to the manufacturer's instructions. RT-PCR will be used to analyze gene expression of *T-bet* (specific for Th1), *GATA3* (specific for Th2), *ROR-γt* (specific for Th17), and *Foxp3* (specific for Treg). The purified RNA will be snap frozen and using GoTaq 2-Step RT-qPCR System (Promega), the RNA will be used to create cDNA and gene expression will be measured according to manufacturer's instructions. To normalize mRNA levels, gene expression of β-actin will be used.

*DNA Binding ELISA:* Estrogen receptor activity will be measured using Trans AM ER DNA Binding ELISAs in 96-well plates. Nuclear extracts from stimulated CD4+ T cells will be assessed for estrogen receptor activity according to manufacturer's instructions.

*Statistical Analysis:* The samples will be performed in triplicate and CD4+ T cells from three separate mice will be tested. Statistical analysis will be performed to determine if BPA and its substitutes significantly alter CD4+ T cell differentiation. The effect of BPA substitutes on estrogen receptor activity will also be determined.

**Timeline**

| **Fall 2022** | |
|---|---|
| Week 1: | Stimulation of T cells |
| Week 2: | Proliferation and Viability assays |
| Week 3: | Stimulation of T cells |
| Weeks 4-6: | Cytokine Detection ELISA |
| Weeks 7-8: | DNA Binding ELISA |
| Weeks 9-13: | RT-PCR |
| Weeks 14-15: | Data Analysis and Progress Report |

| **Spring 2023** | |
|---|---|
| Weeks 1-3: | Data Analysis |
| Weeks 4-5: | Poster Development for Conference Presentation |
| Weeks 6-12: | Writing of Senior Thesis |
| Weeks 13-14: | Oral Defense Preparation |

**Examination Committee**

- Dr. Wade Znosko (Department of Biological and Environmental Sciences)
- Dr. Dale Beach (Department of Biological and Environmental Sciences)
- Dr. Kristian Hargadon (Hampden-Sydney College)

**Figures/Tables**

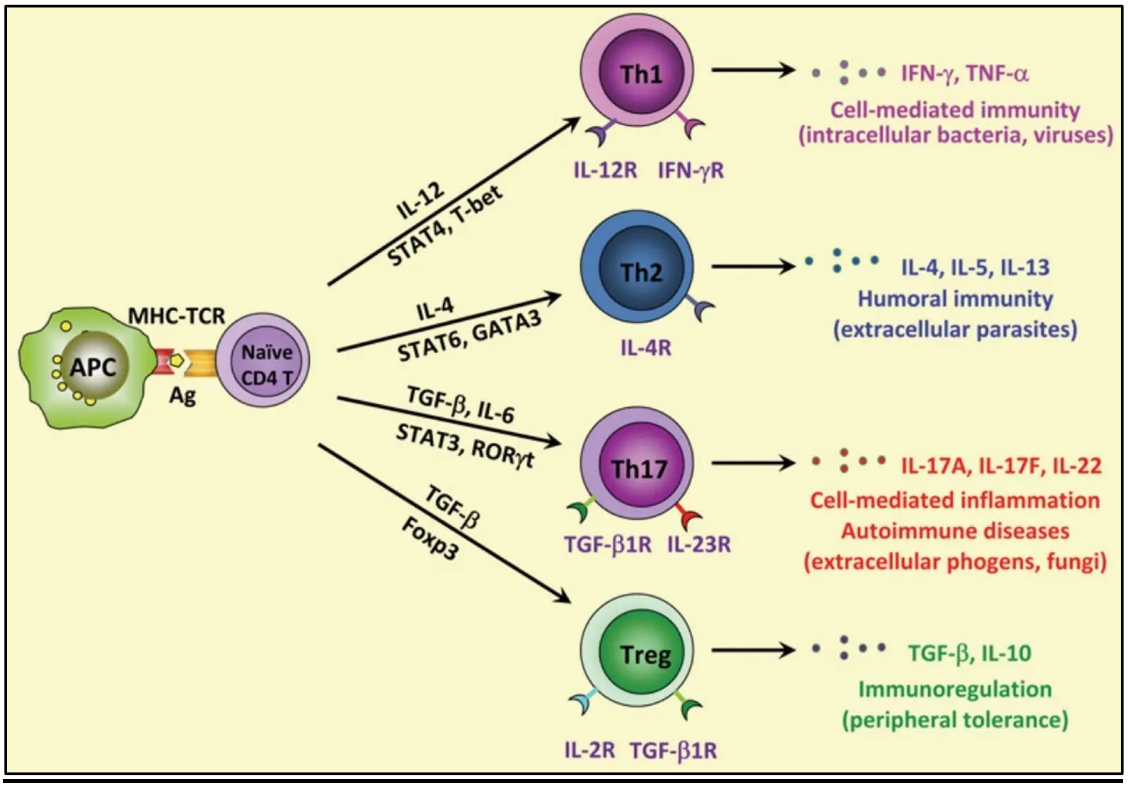


**Figure 1. Differentiation of naive CD4+ T cells.** CD4+ T cells differentiate into Th1, Th2, Th17, and Treg cells, each of which produces different cytokines and has a different function in the immune system.[3]

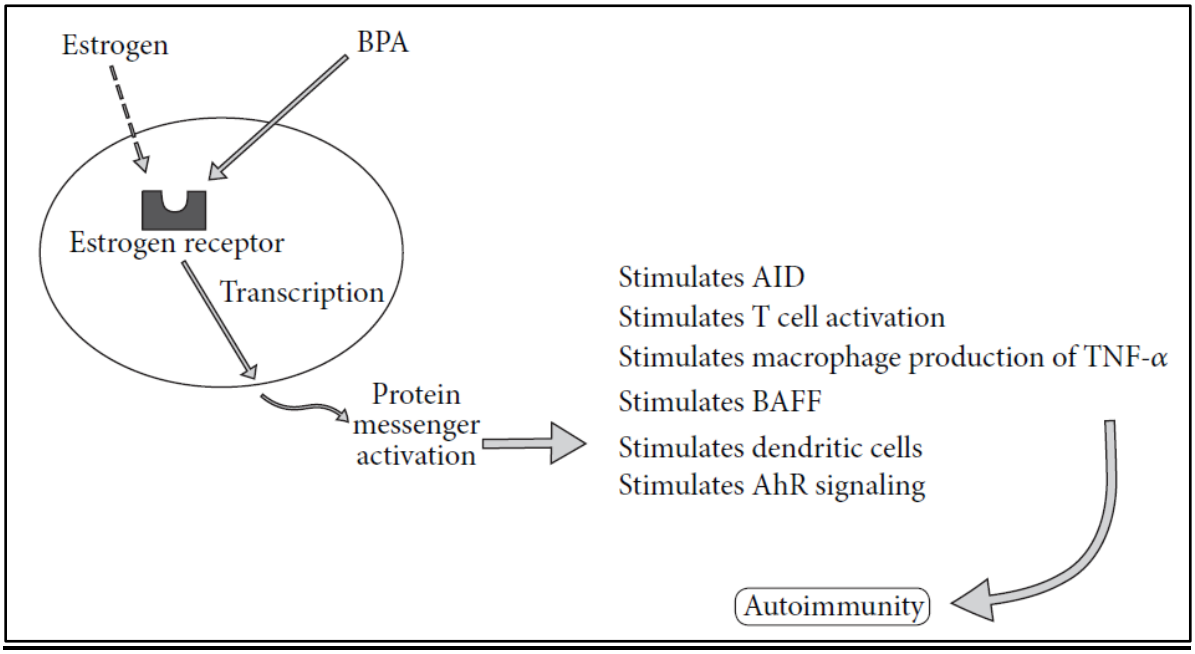


**Figure 2**. **Link between BPA, estrogen receptors, and autoimmune diseases.** BPA can bind to estrogen receptors and influence a number of immune system cells and function, which can lead to the development of autoimmune disorders.[15]

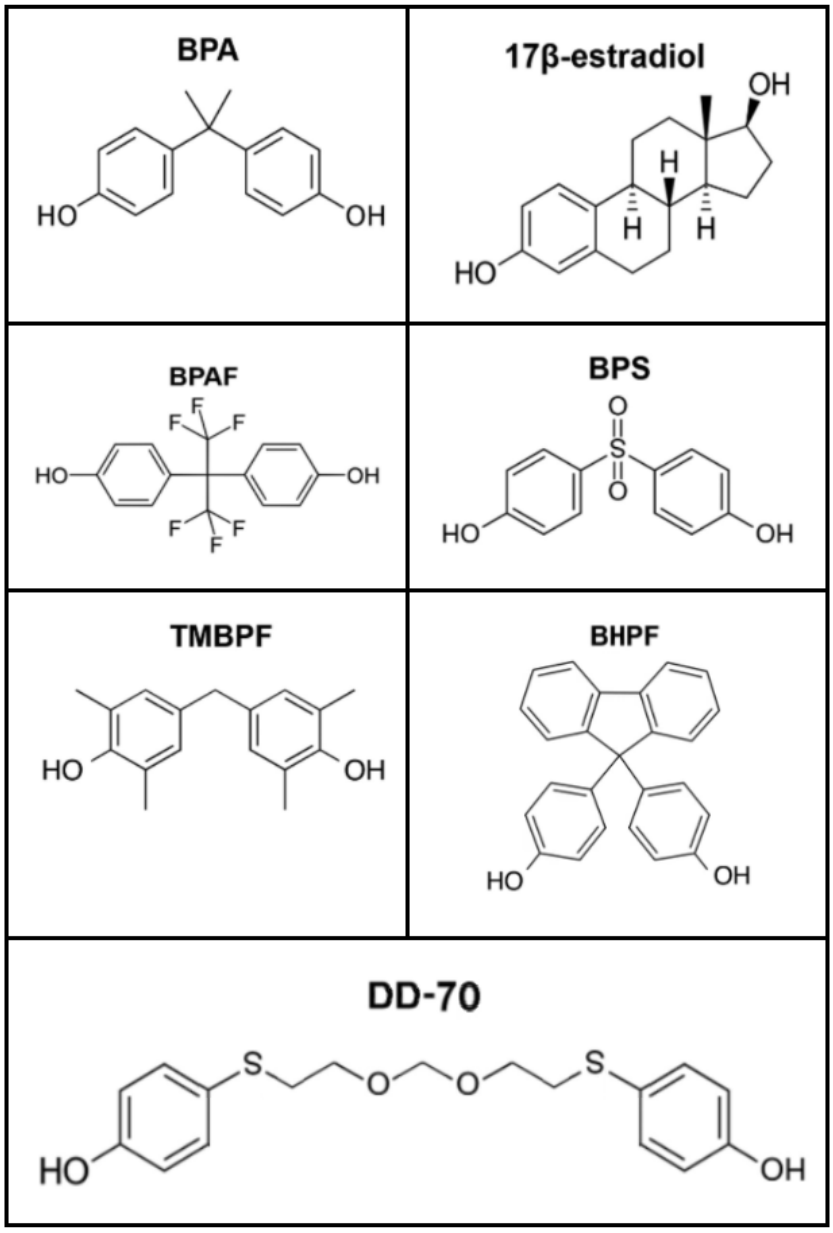


**Figure 3. Structure of bisphenol A (BPA), estrogen (17β-estradiol), and 5 BPA substitutes.** Abbreviations: bisphenol AF (BPAF), bisphenol S (BPS), tetramethyl bisphenol F (TMBPF), 9,9-bis (4-hydroxyphenyl)-fluorene (BHPF), 1,7-bis(4-Hydroxyphenylthio)-3,5- dioxaheptane (DD-70).[14,18]

## References


1. Lionakis M, Hohl T. 2019. Cell-Mediated Defense Against Infection. In: Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. Amsterdam (Netherlands): Elsevier. p. 51–72.
2. Saravia J, Chapman NM, Chi H. 2019. Helper T cell differentiation. Cellular & Molecular Immunology. 16(7):634–643.
3. Leung S, Liu X, Fang L, Chen X, Guo T, Zhang J. 2010. The cytokine milieu in the interplay of pathogenic Th1/Th17 cells and regulatory T cells in autoimmune disease. Cellular & Molecular Immunology. 7(3):182–189.
4. Wan YY, Flavell RA. 2009. How Diverse--CD4 Effector T Cells and their Functions. Journal of Molecular Cell Biology. 1(1):20–36.
5. Khan D, Ansar Ahmed S. 2016. The Immune System Is a Natural Target for Estrogen Action: Opposing Effects of Estrogen in Two Prototypical Autoimmune Diseases. Frontiers in Immunology. 6.
6. Zhang Z, Hu Y, Guo J, Yu T, Sun L, Xiao X, Zhu D, Nakanishi T, Hiromori Y, Li J, et al. 2017. Fluorene-9-bisphenol is anti-oestrogenic and may cause adverse pregnancy outcomes in mice. Nature Communications. 8(1).
7. Roy JR, Chakraborty S, Chakraborty TR. 2009. Estrogen-like endocrine disrupting chemicals affecting puberty in humans--a review. Medical Science Monitor. 15(6):RA137-45.
8. Nomiri S, Hoshyar R, Ambrosino C, Tyler CR, Mansouri B. 2019. A mini review of bisphenol A (BPA) effects on cancer-related cellular signaling pathways. Environmental Science and Pollution Research. 26(9):8459–8467.

9. Liang J, Shang Y. 2013. Estrogen and Cancer. Annual Review of Physiology. 75(1):225–240.
10. Baumgarten SC, Frasor J. 2012. Minireview: Inflammation: An Instigator of More Aggressive Estrogen Receptor (ER) Positive Breast Cancers. Molecular Endocrinology. 26(3):360–371.
11. Siddique S, Kubwabo C, Harris SA. 2016. A review of the role of emerging environmental contaminants in the development of breast cancer in women. Emerging Contaminants. 2(4):204–219.
12. Chakhtoura M, Sriram U, Heayn M, Wonsidler J, Doyle C, Dinnall J-A, Gallucci S, Roberts RA. 2017. Bisphenol A Does Not Mimic Estrogen in the Promotion of the In Vitro Response of Murine Dendritic Cells to Toll-Like Receptor Ligands. Mediators of Inflammation. 2017:1–12.
13. Xu J, Huang G, Guo T. 2016. Developmental Bisphenol A Exposure Modulates Immune-Related Diseases. Toxics. 4(4):23.
14. Ji Z, Liu J, Sakkiah S, Guo W, Hong H. 2021. BPA Replacement Compounds: Current Status and Perspectives. ACS Sustainable Chemistry & Engineering. 9(6):2433–2446.
15. Kharrazian D. 2014. The Potential Roles of Bisphenol A (BPA) Pathogenesis in Autoimmunity. Autoimmune Diseases. 2014:1–12.
16. Sharin T, Gyasi H, Williams KL, Crump D, O'Brien JM. 2021. Effects of two Bisphenol A replacement compounds, 1,7-bis (4-hydroxyphenylthio)-3,5-dioxaheptane and Bisphenol AF, on development and mRNA expression in chicken embryos. Ecotoxicology and Environmental Safety. 215:112140.

17. Maffini MV, Canatsey RD. 2020. An expanded toxicological profile of tetramethyl bisphenol F (TMBPF), a precursor for a new food-contact metal packaging coating. Food and Chemical Toxicology. 135:110889.
18. Harnett KG, Chin A, Schuh SM. 2021. BPA and BPA alternatives BPS, BPAF, and TMBPF, induce cytotoxicity and apoptosis in rat and human stem cells. Ecotoxicology and Environmental Safety. 216:112210.