The Effect of BPC Exposure on Ikaros and RAG-1 Gene Expression In Zebrafish

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Abstract

Background: Bisphenol C (BPC) is a proposed alternative to the chemical additive Bisphenol A (BPA), used in the production of plastics. It is believed to be a safer alternative to BPA, which is known to cause a variety of problems in living organisms following exposure; this can range from the development of breasts in men to cancer and has been linked to the development of autoimmune diseases. This is largely due to the estrogenic activity of BPA. While BPC is classed as a "safer alternative", previous research has demonstrated that BPC has estrogenic activity, like BPA. High estrogen activity itself is linked with predisposition to autoimmune disease. This results in many autoimmune diseases, such as Lupus, that has significantly higher rates of incidence in females than in males. **Methods**: *Danio rerio*, or the common zebrafish, have an immune system similar to that of humans, making them an excellent animal model. Newly-fertilized *Danio rerio* embryos will be treated with varying concentrations of BPC. Embryos will then be sacrificed at varying timepoints and their RNA will be extracted, using phenol-chloroform extraction. Expression of the genes Ikaros and RAG-1 will be quantified by rtPCR and contrasted with untreated embryos of the same age. **Results:** Preliminary results indicate exposure to BPC causes premature upregulation of Ikaros, followed by subsequent upregulation of RAG-1. How early in maturation this premature upregulation occurs appears to be directly correlated to the concentration of BPC the fish are exposed to. **Conclusions:** Our data is still preliminary. Our next step is to solidify the connection between the concentration of BPC and the timeline of Ikaros expression.

Introduction and Research Aims

Bisphenol C (BPC) is a derivative of Bisphenol A (BPA). BPA is an additive in the industrial process of plastic production and has been proven to be toxic by the FDA, resulting in a wide variety of effects, including cancer. Of particular note, exposure to BPA has been implicated in increased risk of developing a variety of autoimmune diseases. Commercial companies use derivatives of BPA like BPC in their products, but various previous research has shown that BPA derivatives are just as harmful. This research specifically looks at the effect of BPC on immune development.

Danio rerio, or zebrafish, are widely used in biological research. It was chosen as the model of this experiment due to its easy maintenance, embryo accessibility, and the similarities it shares with the human genome and immune system. Like humans, zebrafish possess both innate and adaptive immunity, which follows a very similar procedure of differentiation to their human counterparts. Additionally, zebrafish T and B cells differentiate into the same major subclasses as human T and B cells, including Helper and Cytotoxic T cells and antibody producing plasma cells. Plasma cells are also capable of class switching to a variety of antibody subclasses. Thus, zebrafish make an excellent model organism for our project.

The focus of this project is to investigate changes made in the development of the adaptive immune system. In pursuit of that goal, the first two genes investigated are Ikaros and RAG-1, due to their importance in the development of the adaptive immune system. RAG-1 is crucial for the generation of unique T and B cell receptors through the process of VDJ recombination. VDJ recombination is responsible for the variability which allows B cells and T cells to distinguish and react to unique, specific targets called antigens. Ikaros, upstream to RAG-1, is a transcription factor that is responsible for cell differentiation during hematopoiesis. It is crucial in pushing the hematopoietic stem cells towards the lymphoid lineage (which eventually become the B and T cells of the adaptive immune system) instead of towards the myeloid lineage (which is associated with the innate side of the immune system). Furthermore, this project aims to investigate the affect or BPC exposure on B1 vs. B2 cell polarization.

RESEARCH AIMS

- Previous research has shown that BPA acts as an estrogenic compound and signals through both primary estrogen receptors.
- Estrogen is known to exert control over development of both the innate and adaptive branches of the immune system, with elevated levels being associated with abnormal immune development, including autoimmunity.
- We aim to investigate the effect of the BPA alternative BPC on immune development, beginning with adaptive immunity.

Methods

Adult zebrafish (Danio rerio) were bred and embryos were treated separately with BPA and BPC. Embryos treated with EtOH and E3 served as controls. Concentrations of BPC varied but remained within environmentally relevant levels of BPC.

Embryo exposure to BPA and BPC was performed at various timepoints as specified in figures 2,3, and 4. Embryos treated with BPC, EtOH, and E3 were extracted at 0, 1, 3, and 5 hour timepoints. Because Ikaros expression typically peaks at 5 days post fertilization (dpf) and RAG-1 expression at 7 dpf in untreated zebrafish, those times and surrounding timepoints were investigated. Fifty percent water change was performed for experimental groups exposed to treatment longer than 24 hours. Fresh BPC was added after each water change to maintain concentration.

Embryos were harvested after each timepoint and stored in Trizol until cells could be disrupted by bead-beating and RNA could be extracted. rtPCR was run on cDNA to quantify gene expression at each time point.

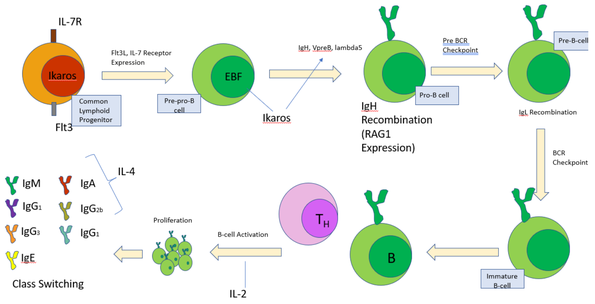

Figure 1. B Cell Maturation
Ikaros commits hematopoietic stem cell to lymphoid lineage. RAG-1 is essential to VDJ recombination in both B cells and T cells. This figure, however, focuses on B cells because the next stage of this study will be focusing on the effects of BPC on B1 vs. B2 B cell polarization.

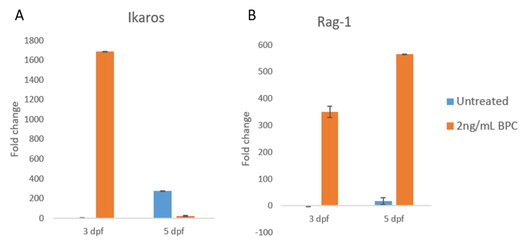

Figure 2. Ikaros and RAG-1 Expression at 3 and 5 dpf
Ikaros (A) and Rag-1 (B) expression in zebrafish embryos either exposed to 2 ng/mL BPC or no BPC, at 3 and 5 dpf. Zebrafish were exposed to BPC immediately after fertilization (0 dpf), with water changes every 24 hours.

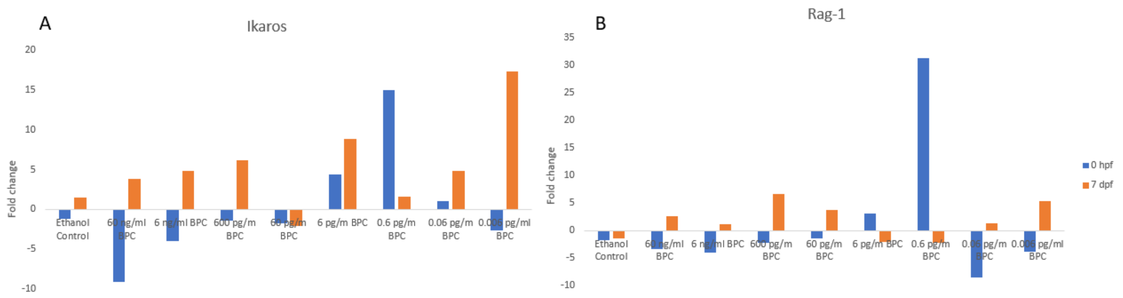

Figure 3. Ikaros and RAG-1 Expression After Exposure to Various Concentrations of BPC
Expression of Ikaros (A) and RAG-1 (B) in zebrafish embryos after 7 dpf, compared with expression immediately after fertilization (0 hpf). Embryos exposed to BPC immediately following fertilization until 7 dpf. BPC concentrations vary from 60 ng/ml to 0.006 ng/ml, and were added directly to the water the fish lived in. Water changes were performed every 24 hours to maintain water quality, with fresh BPC being added with every water change to maintain the appropriate BPC concentration.

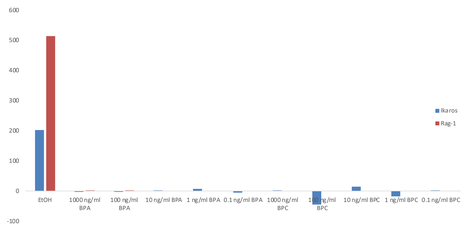

Figure 4. Ikaros and RAG-1 Expression Lost at 10 dpf, Following BPC Exposure.
Ikaros and RAG-1 lost expression after 10 days of exposure, with all concentrations of BPC. Ethanol only control group showed expression of both Ikaros and RAG-1.

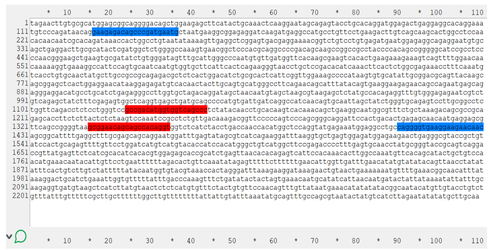

Figure 5. Example Primer Design From a Gene Sequence.
This example displays the Ikaros gene using A Plasmid Editor software. Sequences in blue are the forward and reverse outer absolute quantification primers. Sequences in red are the forward and reverse inner primers. The outer primers are used to generate large products for absolute qPCR. The inner primers are used to produce a smaller product for rt-PCR. Outer primers were manually chosen sequences of 18-24 base pairs. They were then entered into the Oligo Analyzer Tool on the Integrated DNA Technologies website to check for melting temperatures between 50 and 60 °C, GC content around 50%, and Gibbs free energy of forming dimers and hairpin loops. The inner primer sequences were generated by the Primer Blast Tool on the National Center for Biotechnology Information website, and criteria were analyzed with the Oligo Analyzer Tool.

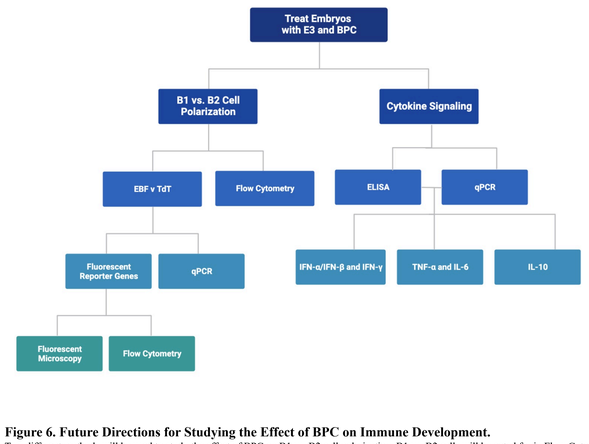

Figure 6. Future Directions for Studying the Effect of BPC on Immune Development.
Two different methods will be used to study the effect of BPC on B1 vs. B2 cell polarization. B1 vs. B2 cells will be gated for in Flow Cytometry using CD19+SPN double positive CD19 positive/SPN cells respectively. B1 B cells express EBF during development but do not utilize TdT during their V(d)J Recombination. Thus, rtPCR will be used to quantify overall EBF and TdT expression in fish exposed to BPC. We expect to see increased EBF expression without accompanying TdT expression, in comparison to their untreated counterparts. Any differences will be confirmed using fluorescent reporter genes. Flow cytometry will be used to quantify numbers of B1 (EBF high/TdT negative) and B2 (EBF high/TdT high) cells. To study the effect of BPC on cytokine signaling, rtPCR of IFN-β ,IFN-α, IFN-γ, IL-6, TNFα, and IL-10 will be conducted following the exposure of BPC. In order to assess the affect of BPC on the immune system's ability to respond to infection, ex-vivo cells, taken from fish either exposed to BPC or not, will be either stimulated by a PAMP, such as LPS, or by viral infection. ELISA and qPCR will be used to assess cytokine expression.

Results and Conclusions

Results:

Based on our earliest experiments, exposure of zebrafish embryos to 2 ng/mL BPC suggested an increased expression of Ikaros, followed by increased expression of RAG-1 (Figure 2). Consequently, a timecourse experiment was conducted with varying amounts of BPC (Figure 3). Contrary to previous thought, results of the timecourse seemed to indicate the opposite of our findings represented in Figure 2, with increased gene expression correlating with lower concentrations of BPC exposure. Therefore, we decided to investigate if increased BPC exposure delayed or prolonged Ikaros and RAG-1 expression by quantifying expression levels at 10 dpf (Figure 4). However, only the untreated controls expressed detectable levels of Ikaros and RAG-1 at this timepoint.

Founded on the combination of this data, we now conclude that BPC does not cause enhanced production of Ikaros and RAG-1, as previously thought, but instead causes premature upregulation of these genes. As with nearly all developmental gene expression oscillations, initial expression levels are undetectable but rise as certain conditions are met. Expression levels will then peak and fall to lower values. This pattern is conserved in Ikaros and RAG-1 expression in zebrafish development, with Ikaros levels normally peaking at 3 dpf and RAG-1 levels peaking at 7 dpf. It is our belief that BPC exposure triggers a premature upregulation of Ikaros, causing expression to peak and begin to fall again before 3 dpf. Likewise, RAG-1 expression would follow a similar curve, found to peak before the usual 7 dpf, due to production of T and B cells in response to premature upregulation of Ikaros. We believe that the reason the data appears to be "backward" in Figure 3 is due to a focus on 7 dpf. As the concentration of BPC dropped, the variable expression curve became closer to the normal expression curve, with Ikaros expression levels still high but dropping and with RAG-1 levels at their peak. However, further experiments must be conducted with varying concentrations of BPC at earlier timepoints to prove the validity of our data interpretation.

Conclusions:

Our data is still highly preliminary, meaning further investigations will be conducted into Ikaros and RAG-1 expression at earlier timepoints, particularly concerning higher concentrations of BPA and BPC. However, our initial conclusions are that BPA and BPC encourage the premature expression of Ikaros and RAG-1, likely by stimulating the estrogen receptor. Specifically, higher concentrations of BPC correlate with an earlier triggering of gene expression.

Future Work

We plan to further investigate how the concentration of BPC relates to the expression time of Ikaros and RAG-1. We will also study other genes, including EBF (early B-cell factor), TdT (terminal deoxynucleotidyl transferase).

We also intend to investigate the effect of Ikaros and RAG-1's premature expression on long-term immune maturation. We are particularly interested in whether the presence of BPC results in a skewing of immune maturation towards a more pro-inflammatory polarization. Thus, we will focus on B1 vs. B2 B cell polarization. It is hypothesized that BPC exposure will increase B1 cell polarization. B1 vs. B2 cell polarization will be studied by using flow cytometry to identify specific surface markers. Furthermore, the expression of EBF vs. TdT after exposure to BPC will be investigated. EBF is expressed in both B1 and B2 cells, but TdT is only expressed in B2 cells. Thus, comparing EBF and TdT expression in the B-cell population will determine B1 vs. B2 B cell polarization.

Additionally, we plan to investigate the effects of BPC on immune signaling. We will also stimulate macrophages in ex-vivo cultures and observe polarization and cytokine production using ELISA. We will concentrate on M1 vs. M2 macrophage polarization and pro- or anti-inflammatory cytokine production. Targeted cytokines include the Type I and Type II Interferons, IFN-α/IFN-β and IFN-γ, respectively; the pro-inflammatory cytokines IL-6 and TNFα; and the anti-inflammatory cytokine IL-10.

References and Acknowledgments

Acknowledgements:

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References:

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