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The Effect of BPA on Nervous System Development in Zebrafish

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Abstract

Bisphenol A (BPA) is a chemical that is commonly used in the creation of plastics and epoxy resins. It can be found in items such as food cans, water bottles, receipts, and dental fillings. Although the FDA states BPA poses no health risk at current exposure levels, there are still concerns about the safety of BPA. BPA exposure may have adverse neurological effects such as preventing estradiol from promoting synapse formation in the hippocampus [9] and has been linked to predisposition to cancer in breast cells [10]. Other previous research has shown likely detrimental effects in zebrafish models such as less developed axons projecting from the spinal cord and a hyperactivity phenotype [4,11]. The goal of this project is to determine the effects of BPA on the development of the zebrafish nervous system by looking at morphological effects in response to BPA exposure. The data in this experiment show that BPA effects pigmentation and body shape in developing zebrafish. In addition, there seems to be a possible effect of BPA on axon formation in the peripheral nervous system. The results of this study will hopefully add to the growing body of knowledge of the detrimental effects of BPA exposure on living organisms, which has both clinical and ecological significance with the growing amount of plastic debris in water systems.

Keywords: Zebrafish, Peripheral Nervous System, BPA

1. Introduction:

Bisphenol A (BPA) is a chemical used in the creation of plastics and epoxy resins. Although the FDA states that BPA is safe at current exposure levels, there is a growing body of evidence that suggests detrimental effects of BPA. For example, BPA has been shown to impair reproductive structures/function [1] and potentially induce oxidative damage in the liver[2]. As shown in Lam et al., 2011 [4], axon branching is negatively affected in zebrafish exposed to BPA [4]. In the same paper, multiple human homologs of zebrafish genes were found to be significantly deregulated in response to BPA exposure.

Because there seems to be many possible effects of BPA on development, the aim of the present study is to add to the evidence for the adverse effects of BPA on nervous system development by using zebrafish (*Danio rerio)* as a developmental model. We do so by providing morphological scores and images of pigmentation and spinal formation as well as images of axon projection from the spinal cord. *We hypothesized that abnormalities in morphology and axonal formation will be associated with BPA exposure in a dose dependent manner.*

Zebrafish were chosen as the developmental model because of several attributes. Most importantly, zebrafish are vertebrates, which makes their developmental process more similar to humans than invertebrate models such as fruit flies. Zebrafish embryos are also transparent until adulthood, which allows internal structures such as the heart and spine to be seen easily. Finally, zebrafish develop quickly and are spawned in large quantities which is convenient for obtaining many samples.

To analyze the morphological data, we used a modified version of a scale developed by Panzica-Kelly et al., 2010 [5] where body shape and pigmentation were scored on a scale from 0-4. Because we saw significant effects on pigmentation, we also quantified the number of pigment cells present on an area of the embryo's head, as done previously by Wiener et al., 2019 [6].

2. Methods

2.1 Fish Husbandry

Tg(olig2:egfp), Tg*(olig2:dsRed), Tg(sox10:nlsEOS),* and **AB* wild type zebrafish (Danio rerio) were obtained from the University of Virginia and bred in the Krasnow Institute of George Mason University. Embryos were produced by pairwise matings, raised at 28.5°C in egg water, staged according to hours or days post fertilization (hpf and dpf, respectively) and embryos of either sex were used for all experiments described below. All animal studies were approved by the George Mason University Institutional Animal Care and Use Committee.

2.2 Drug Treatment

Zebrafish embryos were treated with bisphenol A (BPA) in .5% dimethylsulfoxide (DMSO) mixed in egg water at 0uM control, 10uM BPA, 25uM BPA, or 50uM BPA from 3 hours post fertilization (hpf) high-oblong stage until 48 - 72 hpf long pec to protruding mouth stage. Analyses were performed between 48 and 72 hpf in the high pec-long pec stage. To prevent pigment formation for imaging purposes, embryos were treated with BPA at the same concentration above in .5% DMSO mixed in .003% phenylthiourea (PTU), which replaced the BPA egg water solution after 24 hours.

2.3 Imaging

Embryos were anaesthetized with .003% tricaine by adding one drop of tricaine, waiting for 1 minute, and then mounted on glass-bottom petri dishes with .8% low melting point agarose. Another drop of tricaine was added if embryos were still active after the 1-minute waiting period. Pictures were taken using a mounted camera on an Olympus SD-ILK dissecting microscope. Fluorescent images were taken using a Zeiss AxioZoom. Confocal pictures were taken using a Zeiss LSM after treating embryos with .003% PTU solution to block pigment cell formation. Images were edited with Image J.

2.4 Statistical Analyses

A one-way ANOVA test was used to determine differences between all four groups, with a Bonferroni post hoc analysis. All statistical calculations were done in SPSS.

2.5 Scoring/data collection

Morphological scores were used for determination of abnormalities in pigmentation and body shape. Scores were based on a modified version of the scale described in Panzica-Kelly et al., 2010 [5]. A score of 4 was assigned to embryos with no determinable abnormalities, 3 to mild abnormalities, 2 to moderate abnormalities, 1 to severe abnormalities, and 0 to dead embryos.

Tail coiling behavior was split into two parts: spontaneous and touch evoked. Spontaneous tail coiling was measured by how many tail coils the embryo performed within a 60 second time frame. This was analyzed at approximately 17 hpf. Touch evoked tail coiling was measured by the amount of positive trials present in 5 trials. A trial consisted of 5 pokes to the junction of the back of the head and spine, and a trial was counted as positive if the embryo coiled its tail in response to one of the 5 pokes. This analysis was done at approximately 21 hpf.

2.6 Pigment Cell Quantification

Pigment cells were quantified from the most anterior part of the top of the head to the start of the yolk sac, which is a variation on the method used by Weiner et al., 2019 [6] where they used a triangular area. Embryos were first treated with epinephrine at 5mg/ml for 5 minutes to contract melanosomas, anaesthetized with .003% tricaine, then treated in 4% paraformaldehyde as done in Fernandez del Ama et al., 2016 [13]. This treatment was done at approximately 48 hpf. Pigment cells in the head area of the embryos were manually counted with the cell counter plugin in Image J. Data was then input into SPSS for statistical analysis.

3. Results

3.1 Morphological Effects

Pigment cells are a derivative of neural crest cells. Because peripheral nervous system structures such as axons also arise from neural crest cells, seeing an effect on neural crest derivatives that are not a part of the peripheral nervous system would suggest that BPA may have an effect in early development before PNS formation. After treating the embryos at varying dosages of BPA (0, 10, 25, 50 uM) for 48 hpf, there were easily observable differences in pigmentation between groups. Fig 1a shows that mean pigmentation score decreased when BPA concentration increased. A one-way ANOVA test shows the effect of BPA on pigmentation was significant ($p<.001$, $F=113.361$). Bonferroni post hoc analysis revealed there was a significant difference ($p<.001$) between the control group (n=27, *M*=3.89) and the 50uM BPA group (n=28, *M*=1.04). The graph in Fig 1b shows that the average quantity of pigment cells on the top of the head of the zebrafish also decreased when BPA concentration increased. Again, one-way ANOVA showed there was a significant difference ($p<.001$, $F=24.754$) between groups. Bonferroni post hoc analysis revealed a significant difference ($p<.001$) between the control group (n=10, *M*=42.3) and the 50uM BPA group (n=10, *M*=14.8). Fig 3 shows that the data shown in the graphs is supported by visual observation. In the control group (fig 2a), the pigment cells are numerous and well defined, while in the 50uM group (fig 2c), the pigment cells blend into each other and are less numerous.

Body shape is an indicator of developmental health in zebrafish. Healthy zebrafish should not have significant curvature of the spine in a resting state. However, fig 3 shows that the mean body shape score decreased as BPA dosage increased. One-way ANOVA showed a significant difference ($p=.001$, $F=9.451$) between groups, while Bonferroni post hoc analysis revealed a significant difference ($p=.001$) between the control group (n=27, *M*=3.63) and the 50uM BPA group (n=25, *M*=2.56). This result is visually observable in fig 4a-d, where the control embryo is straight, and the curvature of the spine increases with BPA dosage. Embryos were scored at approximately 48 hpf.

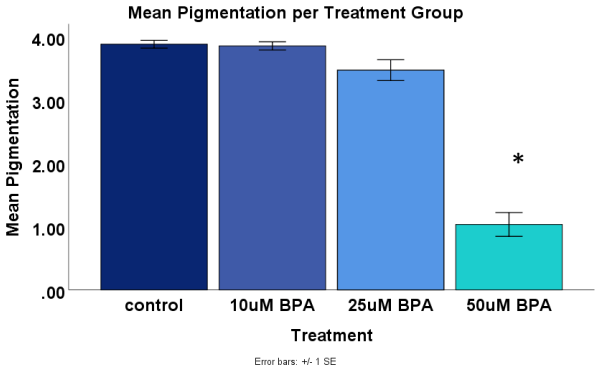

Figure 1a. Bar graph of mean pigmentation per treatment group. Significant differences marked with *.

One-way ANOVA reveals significant differences ($p<.001$, $F=113.361$) while Bonferroni post hoc analysis reveals significant difference between control and 50uM groups ($p<.001$).
Control-10uM p=1.00, Control-25uM p=.205, Control-50uM p<.001 *

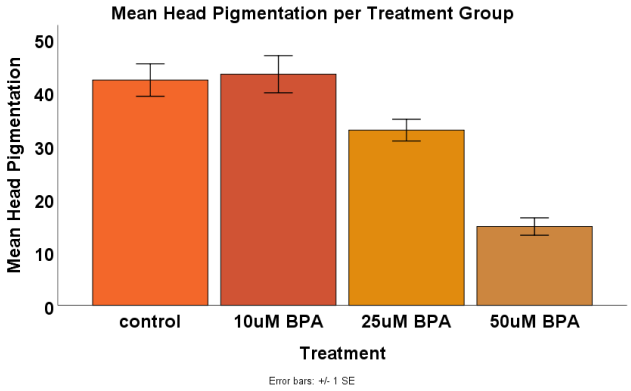

Figure 1b. Bar graph of mean head pigmentation per treatment group. Significant differences marked with *.

One-way ANOVA reveals significant difference between groups (p<.001, F=24.754), while Bonferroni post hoc analysis reveals significant difference between control and 50uM groups (p<.001).
Control-10uM p=1.00, Control-25uM p=.103, Control-50uM p<.001 *

Figure 2a. Head pigmentation of control embryo.

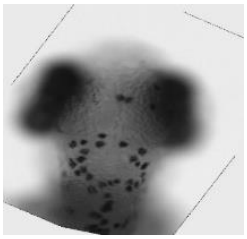

Figure 2b. Head pigmentation of 25uM BPA treated embryo

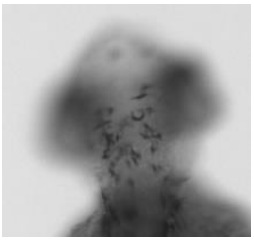

Figure 2c. Head pigmentation of 50uM BPA treated embryo

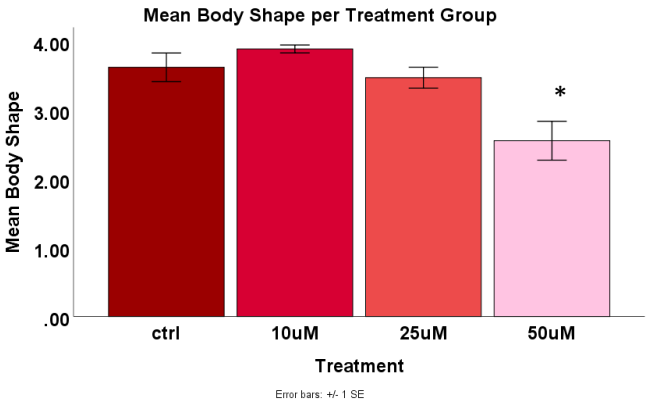

Figure 3. Bar graph of mean body shape per treatment group. Significant differences marked with *.

One-way ANOVA reveals significant difference between groups (p<.001, F=9.451), while Bonferroni post hoc analysis reveals significant differences between control and 50uM group (p=.001).
Control-10uM p=1.00, Control-25uM p=1.00, Control-50uM p=.001 *

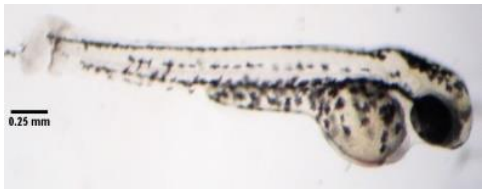

Figure 4a. Control embryo. Body is straight, and pigmentation is well defined.

Figure 4b. 10uM embryo. Body is relatively straight, and pigmentation is relatively well defined.

Figure 4c. 25uM embryo Body is noticeably curved and pigmentation is noticeably less defined.

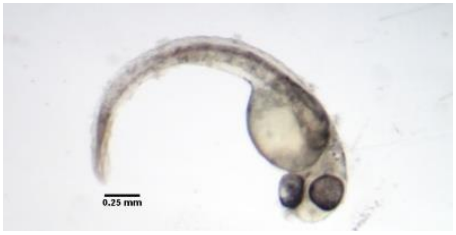

Figure 4d. 50uM embryo Body is very bent, and pigmentation is not very well defined.

Locomotor Effects

Zebrafish start to display tail coiling behaviors at approximately 17-24 hpf. Starting at 17 hpf, zebrafish spontaneously coil their tails. At 19 hpf, zebrafish start to coil their tails in response to a touch to the head or dorsal side of the spine. Because these movements are governed by the peripheral nervous system, deficits in these behaviors may suggest an underlying nervous impact of BPA. Both spontaneous and touch evoked tail coiling behaviors are negatively affected, seemingly in a dose dependent manner. Spontaneous and touch evoked tail coiling decreased with increasing dosage of BPA. A one-way ANOVA revealed that the effect of BPA on spontaneous tail coiling (p=.023, F=3.403) and touch evoked tail coiling (p=.003, F=4.903) was significant. Bonferroni post hoc analysis revealed a significant difference (p=.003) between control and 50uM groups in touch evoked tail coiling but did not reveal a significant difference in spontaneous tail coiling.

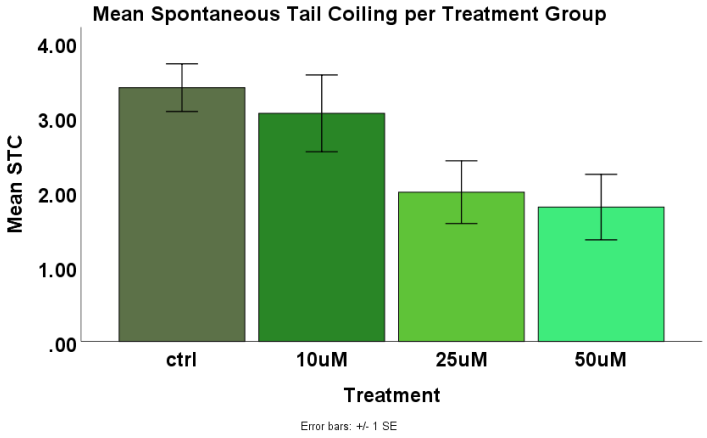

Figure 5a. Bar graph of mean spontaneous tail coiling per treatment group.

One-way ANOVA reveals significant differences between groups (p=.023, F=3.403). However, Bonferroni post hoc analysis shows no significant differences between any pairing.
Control-10uM p=1.00, Control -25uM p=.118, Control-50uM p=.062

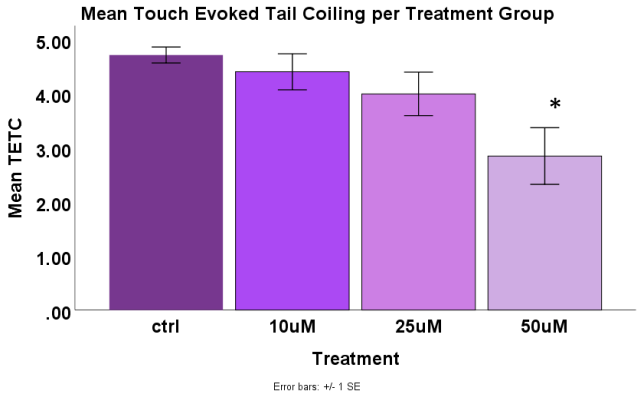

Figure 5b. Bar graph of mean touch evoked tail coiling per treatment group.

Significant differences marked with *. One-way ANOVA reveals significant differences between groups, (p=.003, F=4.903), while Bonferroni post hoc analysis reveals significant differences between control and 50uM groups (p=.003).
Control-10uM p=1.00, Control-25uM p=.889, Control-50uM p=.003 *

Axonal Growth

A role of the peripheral nervous system is to relay the executive information from the brain to the muscles for movement. Because BPA has been previously shown to have adverse effects on motor neuron growth [4], we attempted to replicate those findings. To do this, we took confocal images of motor nerves coming out of the spine in each treatment group at 72 hpf. This was achieved by using transgenic zebrafish that were labeled with a red flourescent marker to visualize oligodendrocytes and a green flourescent marker to visualize Schwann cells [tg(olig2:dsred,sox10:nlsEOS)]. Fig 6 shows a simple diagram of the peripheral nervous system structures of a zebrafish. Fig 7a shows a representative control group image (n=7) of a motor neuron projecting from the spinal cord. In fig 7c, which represents the 50uM group (n=5), an ectopic neuron is seen. It is unclear whether this is a result of BPA exposure, as the sample size is so low, but it is worth mentioning that ectopic neurons were not present in lower treatment groups. Because of the limited sample size of this experiment, more work needs to be done to make a connection between ectopic neuron occurrence and BPA exposure. We hypothesize that deficits would be seen in motor neuron development, and that the deficits will be associated with locomotor deficits, such as the tail coiling deficits seen in this study.

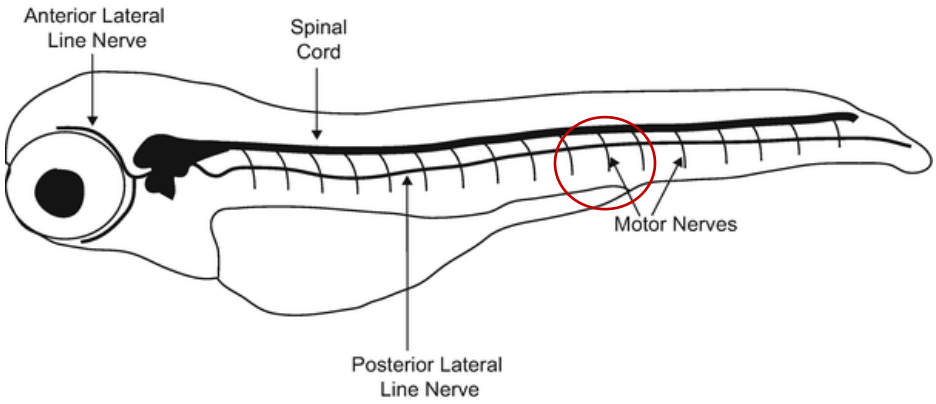

Figure 6. Simple diagram of zebrafish PNS [14]. Imaging was focused on the motor nerves projecting from the spinal cord.

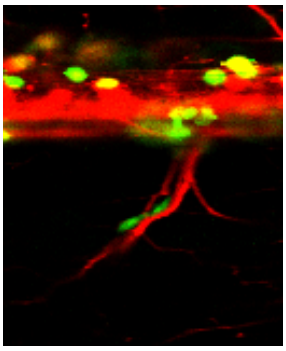

Figure 7a. Confocal image of ctrl zebrafish motor neurons (red) projecting from the spinal cord (large red structure). Control embryos generally had axons that came out of the spinal cord and bifurcated around 1/3 of the way down.

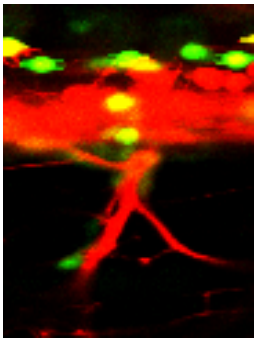

Figure 7b. Confocal image of 10uM BPA treated zebrafish motor neurons (red) projecting from the spinal cord (large red structure). 25uM embryos were difficult to focus in on in the majority of images, but in this particular image, axonal growth seems similar to control, although there may be less myelination.

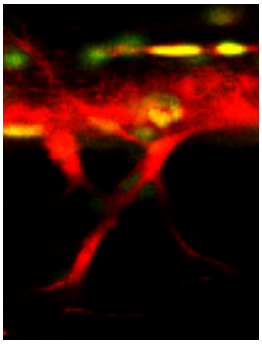

Figure 7c. Confocal image of 50uM treated zebrafish motor neurons (red) projecting from the spinal cord (large red structure). 50uM embryos were especially difficult to focus. In this case, there seems to be an extra axon projecting from the spinal cord (ectopic).

Discussion

The goal of this project was to explore the effect of BPA on nervous system development using zebrafish as a model organism. Overall, the results support the idea that BPA is a potential teratogen with nervous system effects. The mechanism by which BPA exerts its detrimental effects is still being researched, although many studies have show that BPA mimics estrogen [7]. Another study posits that BPA may inflict oxidative damage to cells [2], which could be a result of the estrogen mimicry pathway.

A major finding of this study is the effect that BPA has on pigmentation in zebrafish. As the concentration of BPA rose, so did defects in pigmentation. This deformation was measured on a scale as outlined in Panzica-Kelly et al., 2010 [5]. To reinforce our data, pigment cells on top of the head were manually counted using the cell counter plugin in ImageJ. The results showed a decrease in number and definition of pigment cells as BPA concentration increased. A shortcoming of these findings is the relatively low sample size in the quantitative cell counting group, at n=10 per group. This shortcoming could have avoided had we started to count the pigment cells earlier on in the project when time was more abundant. In the scale experiment, the sample size was approximately n=30 per group, where the differences in pigment cell count were observable as well, but not manually counted.

The results of this study also suggest an effect of BPA on body shape. As the concentration of BPA increased, the curvature of the spine also increased. This was visible in all experiments, especially in the 50uM BPA treatment groups.

Although we did see some changes in motor neuron formation across groups, the sample size was too low to make any conclusions (n<=10). As previously mentioned, the ectopic neuron formation was not seen in any group other than the 50uM group. We predict that there would be a higher incidence of ectopic neurons as BPA dosage increases, and that it would be associated with locomotor deficits, but more work would need to be done to support this hypothesis. The low sample size in this experiment was partly due to the difficulty of getting a focused image. Because the confocal microscope used was sensitive to the micron level, even a slight bend in the specimen would prevent the image from focusing on the desired area and getting a usable image. As the curvature of the embryos increased with the dosage of BPA, so did the difficulty of focusing in on the motor neurons. We will need to find a method to reliably straighten out the embryos to continue this research.

Because of the potential effects on the peripheral nervous system, locomotor abilities were also expected to be affected. In fact, as the concentration of BPA increased, the amount of tail coiling behaviors decreased. However, the differences were only significant between control and 50uM groups for touch evoked tail coiling. Because the one-way ANOVA test found a significant difference between groups, it may be possible that using different post hoc tests would reveal where exactly the significant difference is within the pairings for spontaneous tail coiling. This will need to be investigated further.

Neural crest cells are a common factor in these findings. These neural crest cells migrate from the developing spinal cord and differentiate into many kinds of cells including myelinating cells such as Schwann cells, and pigment cells. Because BPA seems to affect many neural crest derivatives, BPA is likely affecting neural crest cells themselves. The mechanism by which it may happen is not clearly understood, although it is likely that the oxidative [2] and the estrogen mimicking [7] mechanisms are involved.

Although the primary goal of this study was to use zebrafish as a developmental model to translate to humans, the findings of this study are also ecologically applicable, as microplastic pollution is quickly becoming a major threat to aquatic life. In terms of clinical importance, this study shows that BPA will have a teratogenic affect in the developing zebrafish. However, the concentrations used in this study are not representative of the concentrations of BPA humans are exposed to, as the normal serum ranges in humans is around .88-7.0nM [7]. However, there are many studies that suggest a low dosage effect of BPA [15], as well as BPA being more bioavailable in fetal development [16], and so this study serves to add to the growing body of knowledge surrounding developmental effects of BPA.

Acknowledgements

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