The Effects of Four Pure Microplastics on the Development of Zebrafish Cardiac Genes

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ABSTRACT

Microplastics are various chemically composed types of plastic particles that are 5 mm to 10 nm in length. They are now found ubiquitously in marine, estuary, freshwater, and terrestrial environments from pole to pole. It has been well established that many organisms ingest these microplastics. The effects of smaller microplastics, in the nanometer range, after ingestion by organisms is of growing concern. The microplastics used in this study were high density polyethylene (HDPE), polyethylene terephthalate (PET), poly methyl methacrylate (PMMA), and polyurethane (PUR). Previous morphometric studies showed that exposure to these microplastics (specifically PET, PMMA and PUR) had significant changes to total body length and pericardial sac size. The aim of this study is to examine how microplastics may affect certain zebrafish cardiac genes. The particular genes of interest are Nkx 2.5, Tbx 5a and GATA 5. These genes were selected because not only do they have a big impact on embryonic cardiac development, but they are homologs of human cardiac genes. Concentrations of 1 µg/mL and 10 µg/mL of HDPE, PET, and PMMA and PUR were investigated to determine effects on the three cardiovascular developmental genes of interest. RNA isolation, cDNA synthesis, and RT-qPCR techniques were utilized to examine for any genetic alterations. Preliminary results show that PET in 1 µg/mL and 10 µg/mL concentrations and and PUR in 1 µg/mL concentration trended toward an increase in gene expression fold change, which may show that Nkx 2.5 gene is downregulated.

HYPOTHESIS

Exposure to certain microplastics during zebrafish development will cause a change of regulation of Nkx 2.5, Tbx 5a, and GATA 5 cardiac genes compared to the control.

INTRODUCTION

Plastic has become a growing concern over the years as more and more research has shown the detrimental effects of plastics in our oceans. Plastic production has increased astronomically in recent years. "Microplastics" is an umbrella term for thousands of different chemical compounds. When investigating the effects of microplastics on organisms, size as well as composition will be affected. Many of the studies currently focus on microplastics as vectors for plasticizers, persistent organic pollutants, hydrophobic chemicals or biofilms. Very few studies are focused on investigating pure microplastics as individual biochemically reactive polymers (1). This study seeks to further expand on these early studies and are now directing efforts towards explaining how microplastics affect the development of certain zebrafish cardiac genes. The particular genes of interest are Nkx 2.5, Tbx 5a and GATA 5. GATA5 is a regulator gene that aids in the development of ventricular tissue and several aspects of cardiovascular and endoderm development during early stages of embryogenesis. It is also required for the expression of several myocardial genes, such as Nkx2.5 (2). Nkx2.5 is an early myocardial promoter gene required for functional heart formation and development (3). Tbx 5a is a gene associated with the development of several parts of the heart including the atrioventricular canal and heart primordium, as well as the development of the forelimb and central nervous system. Tbx 5a is continuously required for the maintenance of mature cardiomyocyte function (4).

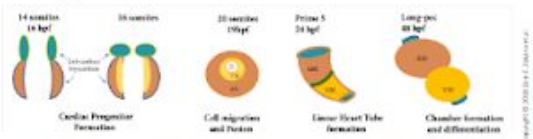

Figure 1. Scheme of embryonic zebrafish heart developmental process

METHODS

Treatment schedule- A sample of 25 AB strain zebrafish embryos were collected per microplastic type and concentration per gene. The embryos were placed into a glass vial with the experimental plastic concentration (1µg/mL or 10µg/mL) in 15 mL of egg water. At 96 hours post fertilization in the treatment, the zebrafish larvae were placed into microcentrifuge tubes, with the treatment removed, and snap frozen in liquid nitrogen and stored at -80°C.

RNA isolation- Rnazol RNA isolation protocol was followed; the sample larvae tubes were thawed and placed in ice. The larvae were homogenized in RNAzol and spun down in a microcentrifuge at 12000 xg for 15 minutes. 600µL of the supernatant was carefully removed and placed into a new tube with 600µL of isopropanol and kept in a -80°C freezer for one hour. The frozen samples were thawed at room temperature for 15 minutes, spun down at 12,000 xg for 10 minutes and the pellet was washed 3 times with 75% ethanol and centrifuged at 8000g for 3 minutes. The ethanol was removed and the samples were dried in a laboratory hood overnight. The sample was then resuspended with 22µL of nuclease free water. Two microliters of the sample were analyzed with nanodrop and the 260/280 absorbance ratio, 260/230 absorbance ratio, and the amount of RNA (in ng/µL) were recorded. The RNA samples were kept in a -80°C freezer.

cDNA synthesis- The High-Capacity Reverse Transcriptase kit: cDNA protocol was followed; the RNA samples were thawed and placed on ice. An aliquot of RT buffer, random primers, dNTP mix, nuclease free H2O, reverse transcriptase and RNA were mixed for a total volume of 10µL per reaction. The tubes were placed into the thermocycler and allowed to run on the ABI-RT setting. The samples were then placed into a 4°C refrigerator.

RTqPCR- The PowerUP SYBR RTqPCR protocols were followed; the cDNA was diluted to 200 nM and 2µL were placed into the microwell plate. An aliquot of PowerUP mastermix, nuclease free H2O, and forward and reverse primers of the specified gene, which were diluted to 500 nM, were mixed for a total volume of 300µL per microplastic type and concentration per gene. The mix was micropipetted into a microwell plate so that the wells contained 20µL of a sample. The samples and controls were run in triplicates to reduce internal error. The plate was spun down at 4000 xg for 5 minutes before being placed into the PCR machine.

Data collection and statistics- The data from the PCR machine was collected from Thermo Fischer Quant Studio 3, and put into Microsoft Excel. The Ct values were averaged for each plastic and gene to obtain the mean Ct. That information was used to determine the expression fold change for each plastic type/concentration and gene and compared to the control.

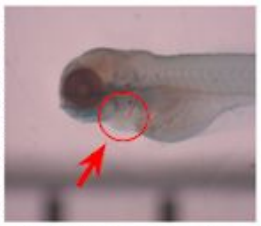

Figure 2: In previous morphometric studies, PET, PMMA and PUR affected pericardial sac size

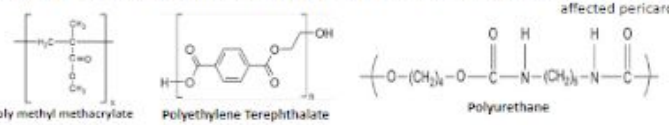

Figure 3. Chemical structures of PMMA, PET, and PUR

RESULTS

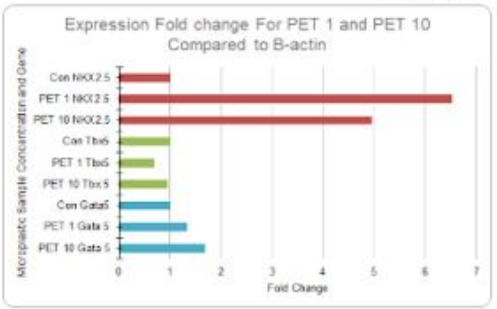

Figure 4: Polyethylene terephthalate (PET) in 1 µg/mL and 10 µg/mL concentrations were examined to see if it affected the regulation of Nkx 2.5, Tbx 5a, and GATA 5 genes. These samples were compared to the housekeeping gene B-actin.

Figure 5: Polyurethane (PUR) in 1 µg/mL and 10 µg/mL concentrations were examined to see if it affected the regulation of Nkx 2.5 and GATA 5 genes. These samples were compared to the housekeeping gene B-actin.

CONCLUSION

To determine if the cardiac gene regulation was affected after exposure to different microplastic types and concentration, the expression fold change was examined. All target gene controls had an fold change of 1. An increased fold change in the target gene experimental would mean that more DNA was needed before a signal was detected by the PCR machine, and therefore a downregulation of gene expression. A decreased fold change would mean that there was more DNA in the sample, therefore an upregulation in gene regulation. There seemed to be an apparent trend based on both figures 4 and 5 of this preliminary study both PET and PUR trended toward an increase in fold change for Nkx 2.5. PET 1 had an average expression fold change for Nkx 2.5 of 6.527 ± 4.839. PET 10 had an average expression fold change for Nkx 2.5 of 4.952 ± 3.844. PUR 1 had an average expression fold change for Nkx 2.5 of 3.122 ± 0.228. This possible downregulation of Nkx 2.5 may affect the transcription factor during embryonic heart formation and development, causing heart malformation diseases. This may be the cause of the change in pericardial sac size in the previous morphometric studies. Another study showed that missense mutations in Nkx 2.5 was common in human patients with congenital heart disease (5). There is a possibility that PET 1 µg/mL and 10 µg/mL and PUR 1 µg/mL may play a role in Nkx 2.5 mutations.

FUTURE STUDIES

The large standard deviation of PET 1 and 10 Nkx 2.5 may be indicative of type I error. At least two more trials must be conducted with PET and PUR to increase the sample size. If those results are similar to the preliminary data, it may show that Nkx 2.5 is actually downregulated by these microplastics. PMMA and HDPE trials must also be conducted. Other cardiac genes will also be investigated in the future, such as GATA 4, Tbx 20, and Hand2 genes.

ACKNOWLEDGEMENTS

Thank you Dr. Lori White, Brittany Karas, Vicky DiBona, Cassie Winz, Michelle Bilotti, Angelica Klejka, Michael Campbell, Chelsea Phuangthong, Daniele Slomko, Shorbon Mowla, Kristin Terez, Eric Shwartzman

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