



Effects of an environmentally relevant PCB mixture on embryonic heart development at HH20 in *Gallus domesticus* (Domestic Chicken)

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INTRODUCTION

Polychlorinated biphenyls (PCBs) are synthetically made chemicals that consist of biphenyl rings with one to ten chlorine substituents. A previous study in tree shrews demonstrated that embryonic exposure to a single PCB congener did not affect survivability, but did affect heart morphology in hatchlings. To examine whether PCBs adversely affected heart development *in ovo*, an environmentally relevant 58-congener PCB mixture was administered to broiler chicken embryos. This study showed that the PCB mixture decreased survivability correlated with increasing concentrations, affected heart rate during embryonic development, and produced a variety of cardiomyopathies in hatching hearts. The effects noted in this hatching study suggested a disruption of protein essential in heart muscle formation during embryonic heart development. To explore this possibility, chicken embryos were exposed to a low and high concentration of the PCB mixture and collected at three significant stages of chick heart development. Hearts were analyzed for abnormalities, proliferative and apoptotic changes, and spatio-temporal distribution of important heart proteins.

SPECIFIC AIMS

- Determine if PCB exposure affects heart morphology at critical stages of embryonic heart development in the domestic chick.
- Determine if PCB exposure affects rates of proliferation and apoptosis in the heart field at a critical stage of heart development.
- Determine if spatio-temporal expression of ventricular myosin heavy chain and titin proteins are affected following PCB exposure *in ovo*.

MATERIALS & METHODS

Broiler chicken (*Gallus domesticus*) eggs were obtained from an Maryland eastern shore hatchery, cleaned, and transported to the University of Maryland, College Park. Eggs were sorted by weight and randomly assigned to treatment groups so that the range and mean of egg weight was similar between all treatments. An environmentally relevant 58 PCB congener mixture made to mimic egg contamination along the Hudson River was used for egg injections. Treatment groups were untreated control, 0.0 µg/g egg wt, low PCB dose (0.08 µg/g egg wt), and a high PCB dose (0.5 µg/g egg wt). PCB doses were selected based on a previous study; the low dose showed no significant lethality and the high dose had significant lethality. Treatment was administered through a small hole drilled into the side of the egg. A micropipettor was used to deposit the volume into the albumen, the hole was sealed with paraffin and the egg was placed on its side into an incubator set at 37°C, 55-65% humidity. Embryos were sampled at HH20, approximately 72 hr of development (Hamburger-Hamilton Staging, 1951). This stage was identified as a significant time point in embryonic heart development. At HH20, the single atrium and ventricle have begun to differentiate, the heart has looped, and proliferation of the cardiomyocytes is prevalent. The heart region was collected, fixed in 4% PFA, and processed for whole mount immunohistochemistry for either Ventricular Myosin Heavy Chain (VMHC) or Titin (*primary antibodies obtained from DBHB*). These antibodies were selected due to their exclusivity in the heart field at this stage of development and their known function during cardiac development. VMHC protein is important to the proper function of the compact layer of the heart wall and titin is a protein necessary for myofibril connections. Following whole-mount imaging, hearts were frozen in gelatin, and sectioned. Heart sections were then assayed for proliferation (using a *phospho-Histone-H3 antibody* (pHH3)) and apoptosis (using a *Roche TUNEL kit*) within the heart field, and nuclear stained with DAPI. Embryos were compared across treatments for lethality, proliferation and apoptosis rates, cardiomyopathies in whole mount and on sections, and area of the heart field. A minimum of eight sections was analyzed for average data collections. Lethality and whole mount abnormalities were statistically analyzed using a Chi square test. Proliferation averages were analyzed by one way ANOVA followed by Tukey's post-hoc test.

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RESULTS

- Developmental exposure to an environmentally relevant 58 congener PCB mixture affects survivability in both the low dose and high dose treatment (Chi square: $p < 0.001$, Figure 1).
- Exposure to the 58 congener PCB mixture increased heart abnormalities in the low dose treatment in the surviving embryos (Chi square: $p = 0.08$, Figure 2).
- Proliferation of cells decreased in the heart field in embryos exposed to the 58 congener PCB mixture, with a significant effect at the high dose (Tukey's: $p < 0.001$, Figure 3).
- Exposure to the PCB mixture did not affect apoptosis of cells in the heart field.

DISCUSSION

Exposure to the 58 congener PCB mixture affected survivability at both the low dose and high dose treatments (Figure 1). In a previous study using the same doses, survivability at hatch increased at the low dose treatment but was not significantly different associated with the small sample size (Carro, 2009). This study demonstrated that there was an increase in heart abnormalities in both PCB treatment groups when compared to the controls at embryonic HH20, with a greater percent of abnormalities at the low dose (Figure 2). It can be assumed that the most severe cardiomyopathies will result in embryonic death, while chicks may survive less severe abnormalities (Kirby, 1995). This may account for the increased abnormalities seen in viable embryos at the low dose at HH20. Proliferation decreased significantly in the heart field at HH20 (Figures 3 & 5), while no difference in apoptosis was found. At HH20, proliferation of the cardiomyocytes is essential in creating proper heart wall morphology. This lack of proliferation might account for the abnormalities observed in hatching hearts (Carro, 2009). No difference was observed in the spatio-temporal expression of VMHC and Titin proteins, two heart specific proteins essential in the proper formation of the heart at HH20. The analysis of these proteins did not show any differences in spatio-temporal expression; however potential follow-up should consider mRNA expression or additional heart specific proteins (Figures 4 & 5). In conclusion, the major effect of PCB exposure was a decreased proliferation in the heart field.

Figure 1: Percent survivability of fertile broiler chicken embryos at HH20 (72hrs) of embryonic development

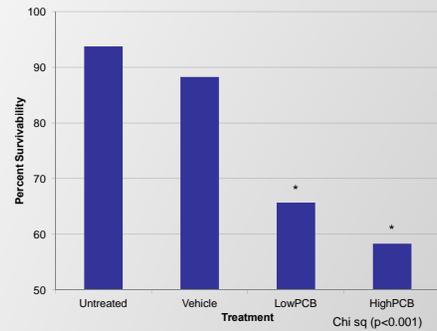


Figure 2: Percent abnormalities in hearts of fertile broiler chicken embryos at HH20 (72hrs) of embryonic development

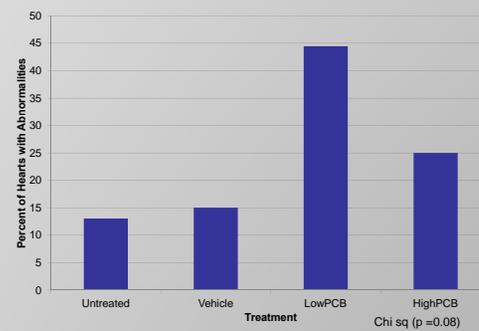


Figure 3: Average number of proliferating cells in broiler chick embryonic heart field at HH20 (72 hours) of embryonic development

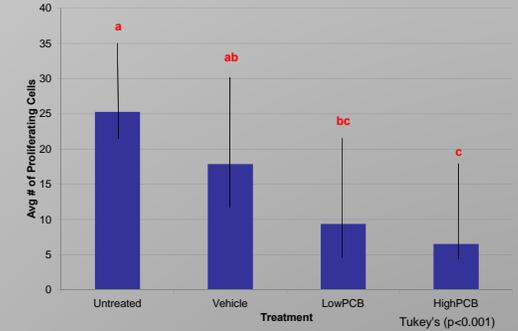
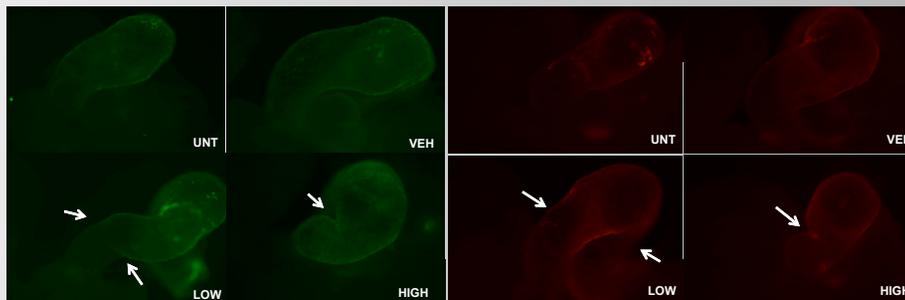
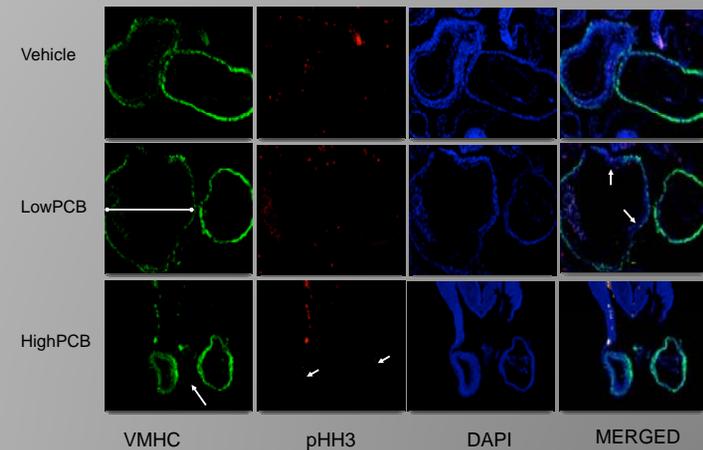


Figure 4: Immunohistochemistry of HH20 chicken embryos using VMHC (green) and Titin (red) primary antibodies (Images captured using a 5x objective).



White arrows indicate heart abnormalities. Low dose treatment shows incorrect looping pattern and narrowing of the heart tube. High dose treatment shows heart wall deformity. Specific abnormalities were not identified exclusively in either treatment.

Figure 5: Immunohistochemistry of HH20 chicken embryonic heart sections (14µm). GFP-labeled Ventricular Myosin Heavy Chain (VMHC) protein, DS-Red-labeled anti-phosphohistoneH3 (pHH3), DAPI nuclear staining (blue).



VMHC: white arrows indicate abnormal heart field. pHH3: white arrows indicate lack of proliferation in the heart field. Merged: arrows indicate proliferation is not within heart field.