DOES PERCHLORATE INDUCE NON-ALCOHOLIC FATTY LIVER DISEASE IN DEVELOPING ZEBRAFISH?

By

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Abstract

Perchlorate is a chemical contaminant found in various food and water sources. Millions of Americans and individuals around the world are exposed to perchlorate primarily from military and engineering manufacturing wastes[1]. Previous research has shown that perchlorate causes hypothyroidism in vertebrates, including people[2]. Stickleback fish (*Gasterosteus aculeatus*) have displayed hypothyroidism and signs of non-alcoholic fatty liver disease (NAFLD) when exposed to perchlorate[3]. NAFLD is excess fat deposits in liver tissue that can lead to various negative health outcomes such as liver cancer or liver failure[4]. One hundred and fifty-five zebrafish (*Danio rario)* were placed into four exposure groups: control, 10 ppb, 10 ppm, and 100 ppm of perchlorate. Between 100-115 days post-fertilization, the zebrafish were euthanized and fixed in 10% buffered formalin. The tissues were processed and embedded in paraffin wax, sectioned, and treated with H & E staining[5]. The samples were analyzed at 40x magnification and LASX software was used to calculate total lipid area droplet size and roundness and number of lipid droplets and median droplet size were calculated with these data. The data for each of the treatment groups were compared using a one-way ANOVA. The results indicate that perchlorate exposure does not impact the total lipid area, median droplet size, or number of lipid droplets in zebrafish. The difference in response to perchlorate exposure and liver tissue in zebrafish and stickleback fish may be due to genetic or environmental differences in the species[6].

Introduction

Perchlorate is a synthetically made chemical that also occurs naturally at low concentrations in arid regions [7]. It is used in the manufacturing of airbags, fireworks, and solid rocket propellant [7, 8]. In an analysis of the urine of 2,820 Americans ages six years and older, perchlorate was found in all samples [9]. Therefore, perchlorate is a ubiquitous contaminant of people and the environment.

Perchlorate exposure can induce non-alcoholic fatty liver disease (NAFLD) in developing stickleback, but perchlorate exposure does not cause the same disease in zebrafish [3, 6]. NAFLD is characterized by an excess of fat in liver tissue that can result in scarring of the liver, cirrhosis, or liver failure [4]. According to a National Health and Nutrition Examination Survey (NHANES) conducted from 1999 to 2014 with 29,094 participants, NAFLD prevalence was associated with samples containing urinary perchlorate[10]. This association was

significant when comparing the levels of urinary perchlorate and the hepatic steatosis index (HIS) and the US fatty liver index (USFLI). Both indexes are used to assess an individual's risk and the severity of NAFLD. These findings demonstrate that perchlorate can induce a range of adverse outcomes on the liver, and that these outcomes vary by taxa.

Materials and Methods

Sample preparation

Ten adult female and ten adult male zebrafish were put in 37.85 L tanks with DI water mixed with 1.0 g/L Instant Ocean© solution at 20 L/tank or 2 L/individual. The zebrafish were separated by sex. The fish were fed 175 mg of 300-micron GEMMA MICRO food once a day. The light:dark photoperiod was 10:14 and the temperature of the water was 24 degrees C°. The tanks were cleaned weekly and, every two weeks, the carbon filters were replaced in the tanks. Ninety percent of the water volume was replaced every week for cleaning and tanks were scrubbed to remove any debris or algae.

The zebrafish were fed 175 mg of 300-micron GEMMA MICRO food twice a day,12 hours apart with supplemental shrimp feedings to prepare for breeding. The photoperiod of light:dark was shifted to 14:10 and the water temperature was raised to 28 C°. The zebrafish were brought into a breeding tank with three males and four females in each tank. The fish were left overnight and then a light was turned on in the tank. The fish were removed to search for viable embryos three hours after the light turned on. The adult fish were immediately euthanized after being transferred to 37.85 L tanks with a water volume of 200 mL per fish. All of the viable embryos were assigned an exposure group and rinsed with an antifungal solution at least four times. They were then placed on sterile glass Petri dishes containing water either without perchlorate (control group) or with perchlorate (concentration). The experiment included a control treatment group without perchlorate exposure as well as three treatment groups of 10 ppb, 10 ppm, and 100 ppm concentrations. The embryos were monitored until they hatched and then were evaluated for swimming performance. The fish were euthanized using Tricaine and fixed in a 10% buffered formalin solution for thirty minutes. The fish were taken out, placed in the formalin a second time, then put into a 75% alcohol mixture for 20 minutes and then into another 90% alcohol mixture for thirty minutes. The leftover alcohol in the 95% mixture was added to the samples for another 30 minutes. The samples were put in ethanol and xylene with 100% alcohol for thirty minutes and the embedding machine was turned on. Then, the blocks were put into a new 100% alcohol mixture for thirty minutes and into a second xylene mixture for thirty minutes. The blocks were placed into a

third xylene mixture for 30 minutes, paraffin wax for 45 minutes, taken out, then another 40 minutes in wax. Lastly, the blocks were placed in wax for one hour[5]. The samples were cut into 10 μm coronal sections and stained with hematoxilyn and eosin (H&E). Each sample was separated onto six slides and between four to six sections were on each slide. Each sample was assigned a numeric code to keep experimenter blind to treatment group.

Image Analysis

Slides were analyzed using a Leica microscope with LASX software. A total of 20 samples were analyzed from the control group, 19 from the 10 ppb treatment group, 19 from the 10 ppm treatment group, and 20 from the 100 ppm treatment group. The measurements aimed to determine if perchlorate can induce NAFLD in zebrafish. To quantify the association, the software was used to determine median droplet size, total lipid area, number of lipid droplets, and sum of lipid area. An image was taken of the liver of each sample on the software. Area of the tissue on the image varied on the size of the liver tissue in each sample. After the image was taken, the software measured the area, length, perimeter, roundness, and shape factor.

Results

Perchlorate exposure was not significantly associated with non-alcoholic fatty liver disease in zebrafish. Fig. 1 compares liver tissue found in each treatment group.

Fig. 1

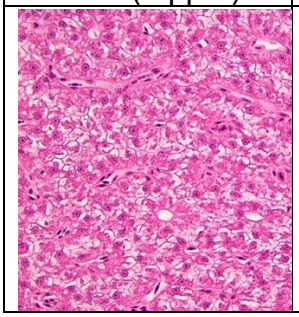
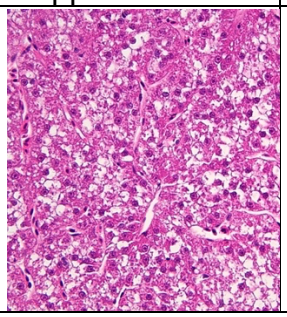
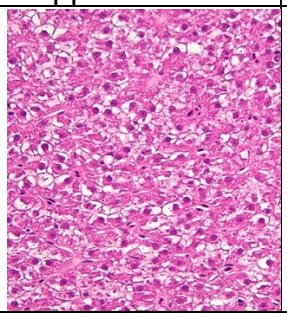
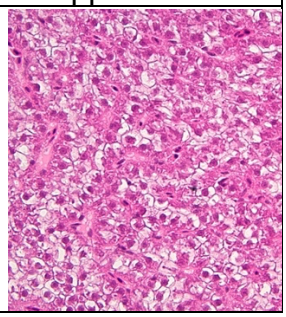
Control (0 ppm)	10 ppb	10 ppm	100 ppm
			

Fig. 2 shows the mean total lipid area from the samples for the control group was 1782.5 μm^2, 1400.4 μm^2 for the 10 ppb group, 2116.5 μm^2 for the 10 ppm group, and 1727.7 μm^2 for the 100 ppm group.

Fig. 2

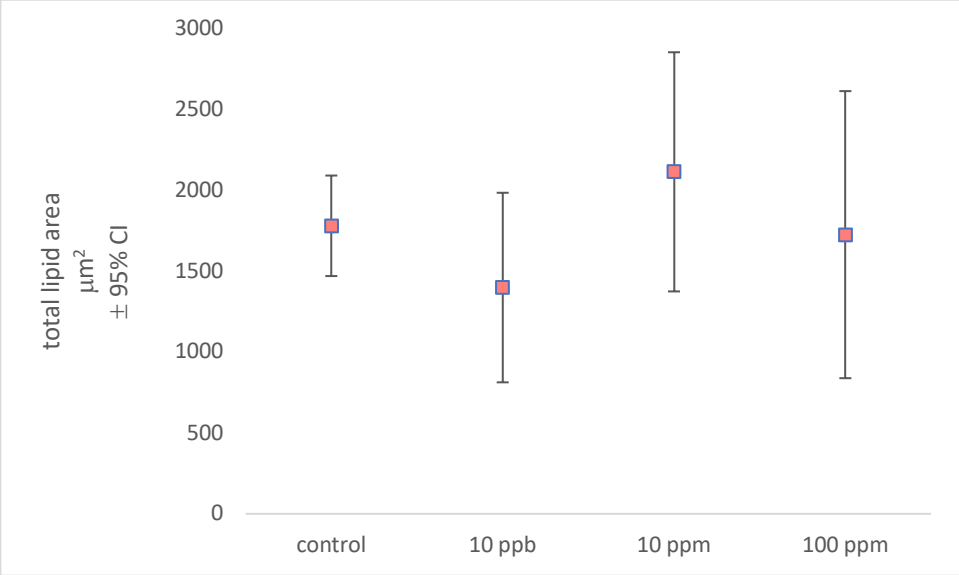


Fig. 3 represents the mean number of lipid droplets with the control group having 427.9, 283.3 for the 10 ppb group, 531.2 for the 10 ppm group, and 459.5 for the 100 ppm.

Fig.3

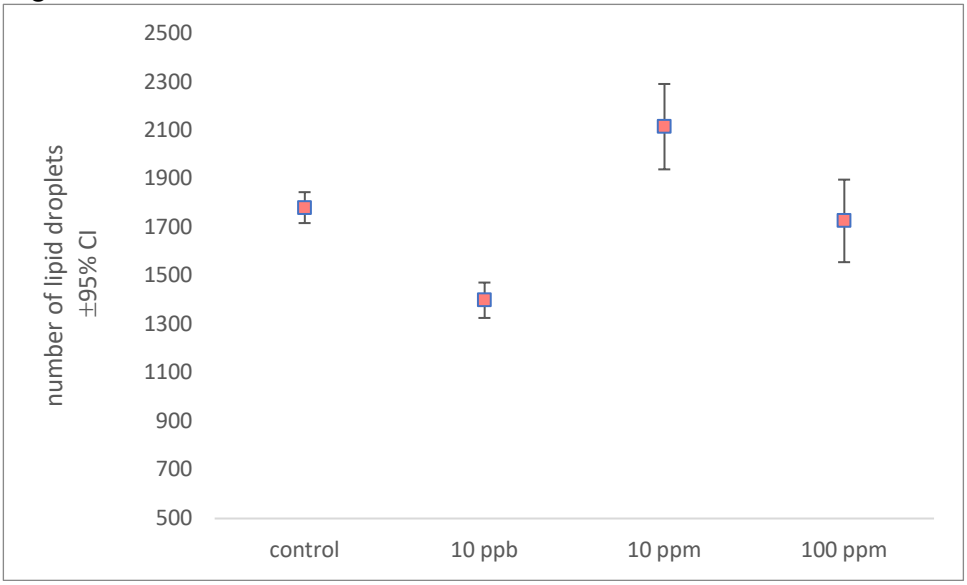
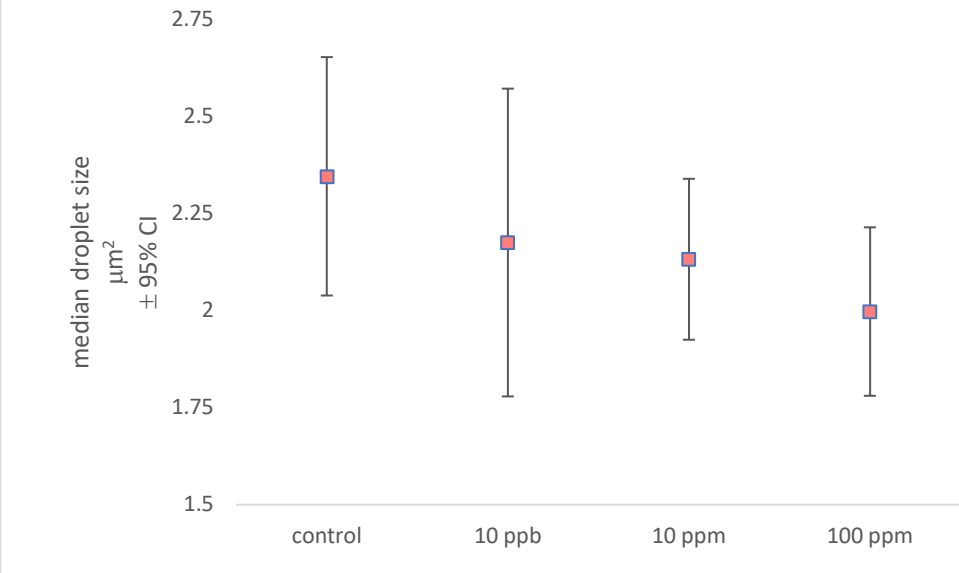


Fig. 4 exhibits the median droplet size of each treatment group with 2.347 μm^2 for the control group, 2.176 μm^2 for the 10 ppb group, 2.133 μm^2 for the 10 ppm group, and 1.998 μm^2 for the 100 ppm group.

Fig. 4



Discussion

Perchlorate exposure is not associated with non-alcoholic fatty liver disease in zebrafish. The median droplet size, the total lipid area, and the number of lipid droplets did not vary significantly across treatment groups. This reinforces the findings in zebrafish of Minicozzi et al.[6], and differs from the findings of perchlorate exposure in stickleback fish by Minicozzi et al. [3],[6]. Stickleback and zebrafish may differ in their vulnerability due to genetic factors. For example, even control groups have more lipid droplets in stickleback fish. Another explanation for the differing responses to perchlorate exposure between zebrafish and stickleback fish may be because of their evolutionary and environmental differences[6]. Zebrafish are found in tropical and lower latitude water whereas stickleback fish are native to higher latitude, arctic waters [6]. This may give an evolutionary advantage for stickleback fish to be able to accumulate lipid droplets.

Conclusion

Developing zebrafish exposed to perchlorate did not present with characteristics of non-alcoholic fatty liver disease. There was little difference in

the averages across treatment groups for the median droplet size, total lipid area, and number of lipid droplets. These results are consistent with previous experiments evaluating the effects of perchlorate exposure and zebrafish[6].

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