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Effects of PPA on zebrafish

Biology

The effect of phenylpropanolamine on the development of the lateral line system, heart rate, and morphology of *Danio rerio* larvae.

Abstract

Phenylpropanolamine (PPA) is a sympathomimetic drug that is a vasoconstrictor and bronchodilator. It was a component of cold and cough medications, however in 2000, it was determined that it caused stroke and was therefore removed from the market. There have been various studies on the effects of PPA on the body, however research on a relationship between PPA exposure and hair cell development has not yet been observed. A useful organism to study ototoxic effects is the zebrafish (*Danio rerio*) because the lateral line system along the outside of their body is composed of hair cells. Due to similarities between the hair cells of the lateral line system and the hair cells of the mammalian inner ear, ototoxic effects on the hair cells of the lateral line can allude to effects of the hair cells of the mammalian inner ear. When motion in the water stimulates the lateral line, a signal is sent to the nervous system and an escape response occurs. Using this knowledge, tests were done to stimulate lateral line in zebrafish larvae and determine if there was a difference in the reaction times of larvae which developed until 5 days post fertilization (dpf) in various concentrations of PPA ranging from 0.25 to 1.5 mM. Additionally, heart rate and morphology were observed after incubation in PPA and heart rate was observed again after an over-night recovery. Reaction time results were determined to be inconclusive. However, heart rate results showed that there was a significant decrease in the heart rate of larvae treated with 0.50 and 0.75 mM PPA until 5dpf. After recovery, larvae treated with 0.25 and 0.50 mM PPA experienced a significant increase in heart rate which was not significantly different from the control. Differences in morphology were observed among concentration groups.

Keywords: cardiovascular system, edema, PPA, recovery, zebrafish

Phenylpropanolamine (PPA) is a drug similar to amphetamines that was found in many over-the-counter cough and cold medications (Cantu et al. 2003). It was also used as an over-the-counter appetite suppressant (Hsieh et al. 2007). However, it was removed from the market of cough and cold medicine in 2000 due to episodes of stroke following its ingestion (Flavahan 2005). PPA is a sympathomimetic amine, meaning that it mimics the effects of neurotransmitter substances on the sympathetic nervous system. PPA binds to both α_1- and α_2-adrenoreceptors which are found on vascular smooth muscle and trigger vasoconstriction (Flavahan 2005). A study done by Suwalsky et al. (2011) determined that PPA also affects the shape and structure of the membranes of human erythrocytes. Despite the attempt to remove PPA from pharmaceuticals, it is still used in veterinary medicine as a treatment for urethral sphincter mechanism incompetence (Claeys et al. 2011) and it may also be present in homes of people that were not aware of the recall (Delorio 2002).

Previously, the effects of PPA on a developing nervous system of zebrafish embryos were studied by Anna Galle (2013), in which she determined that the touch response of larvae treated with 2mM and 4mM solutions of PPA were significantly affected. However, the effects on the developing lateral line system have not been studied. *Danio rerio* (zebrafish) are great models for toxicology. Since so much is known about zebrafish, they can be very useful to determine adverse effects due to drugs such as PPA. Zebrafish have been used to study systems such as the cardiovascular, muscular, and lateral line. The lateral line system of zebrafish larvae is used to sense and escape the strike of a predator (McHenry et al. 2009). The larvae can sense the flow of water with receptors called neuromasts which are dispersed over the length of the body (Trump and McHenry 2008). Under natural development and structure, stimulation of the

lateral line causes an escape response in the larvae. However, if there are any defects in the lateral line system the escape response may not be evoked or may be delayed.

It is important to learn the effects of PPA on development in model organisms such as the zebrafish in order to allude to effects that it may have on human development. Connections can be made due to similarities between biological systems in zebrafish and in humans. For example, the hair cells of the neuromasts of the lateral line system in the zebrafish are similar to the hair cells in the mammalian ear (Chiu et al. 2008). Hair cells in the mammalian ear respond to mechanical stimulation such as sound, angular acceleration, and linear acceleration. If damaged, mammals may lose the ability to hear and balance. Due to similarities between the hair cells of the mammalian ear and hair cells of the neuromasts, drugs may have similar effects on either structure. Various studies have found that a variety of environmental toxicants including metals and pharmaceuticals affect neuromasts development and/or function (Froehlicher et al. 2009). To learn more, I investigated the effects on the stimulation of the lateral line system and escape response of *Danio rerio* larvae due to development in solutions of PPA. The experiment tested the hypothesis that higher concentrations of PPA would affect the lateral line system and therefore cause the escape response to be delayed or absent. Measured the escape response reaction time by stimulating the lateral line the of zebrafish larvae which were incubated in various concentrations of PPA. In addition, other observations such as heart rate recovery and morphology were also examined.

Methods

Embryo Collection and Treatment with Phenylpropanolamine

OSU-5D adult zebrafish from the Ripon College Biology Department were crossed, embryos were collected and placed into petri dishes with a layer of 1X egg water and incubated at 28°C. The lateral line begins to develop around 18 hours post fertilization (hpf) when the first placode appears and gives rise to neruoblast precursors (Pujol-Martí and López-Schier 2013). Therefore, around 18 hpf the zebrafish embryos were screened for survival and exposed to solutions of PPA. Twenty embryos were exposed each PPA solution with concentrations of 0.25 mM, 0.5 mM, 0.75mM, 1.0mM, and 1.5mM, in individual wells of a six-well plate. A control group of 20 embryos developed in 1X egg water (Westerfield 2000).

Lateral Line Assay

All embryos were incubated at 28°C until 5 days post fertilization (dpf) because this is when the lateral line is fully functional and integrated with the motor system (Liao 2010). This ensured that any reaction results from stimulation their lateral line would be due to the effects of PPA, and not due to under development of the lateral line system or poor integration with the motor system. The 5 dpf larvae were removed from the solutions of PPA, placed in a petri dish with 1X egg water under a Leica EZ4 dissecting microscope connected to a camera and screened for survival. Stimulation of the lateral line system was done by dropping a 0.2310 g glass bead into the water from 10.16 cm above the petri dish. The response of each larva was videotaped using the program LAS EZ. Using the video viewer QuickTime, the response time of each larva was determined. This data was entered into Microsoft® Excel and analyzed using a two sided *t*-test to determine the effect of PPA on their response time. Regression analysis was also performed using Microsoft® Excel.

Recovery Assay

The ability of the larvae to recover after being exposed to PPA was also studied. PPA is known to be a vasoconstrictor in humans, and therefore has an effect on the cardiovascular system. To study their recovery, heart rate was analyzed during and after exposure to PPA. Ten embryos were exposed to each concentration of 0.25mM, 0.50mM, and 0.75mM PPA at 18 hpf, while a control group of 10 larvae were incubated in 1X egg water. Larvae were removed after 5 dpf and were placed in a petri dish with 1X egg water under a Leica EZ4 dissecting microscope connected to a camera. Photographs were taken of 5 of the 10 larvae in each concentration, which were then treated with 0.017% tricaine methanesulfonate to reduce their movement. Heart rate was determined by counting the amount of heart beats in 30 seconds, was then converted to beats per minute and recorded. The remaining 5 untreated larvae of each concentration group were immediately returned to 1X egg water and incubated at 28°C overnight. The following day each larva was treated with tricaine, and their heart rates were once again recorded. Heart rate data was entered into Microsoft® Excel and a *t*-test was done to determine if there was significant change in heart rate.

Results

Survival

Survival was observed at 5 dpf (Table 1). One hundred percent of control group and 0.50 mM PPA larvae survived until 5 dpf. The 0.25mM PPA solution resulted in 95% survival and the 0.75 mM PPA solution resulted in 85% survival after 5 dpf. Concentrations of 1.0mM and 1.5 mM PPA resulted in 0% survival after 5 dpf.

Reaction

Larvae incubated in 0.25 mM and 0.50 mM PPA were all able to quickly react when the bead was dropped. However, although alive, larvae incubated in 0.75mM PPA did not react at all. A statistical *t*-test determined that there was a significant difference between reaction times of control and 0.25 mM PPA groups (P=0.004) and 0.75 mM PPA groups (P=1.1E-7) However, there was no significant difference between control and 0.50 mM PPA groups (P=0.14) (Figure 1).

Regression analysis of the observed reaction times of each group of larvae determined that there was not a strong relationship among reaction times of each group. The r^2 values of the control, 0.25 mM, and 0.50 mM PPA groups were 0.56, 0.09, and 0.00008, respectively.

Heart Rate Recovery

A difference in heart rate was observed between each group of larvae. After the larvae were incubated in PPA, the heart rate of the larvae was slower with increasing concentration of PPA. There was a significant difference between control and 0.50 mM and 0.75mM PPA groups (P=0.034 and 0.00063, respectively), however there was no significant difference between the control and the 0.25 mM PPA group (Figure 2). After larvae were allowed time to recover in 1X egg water overnight, an increase in heart rate from before recovery was observed in each experimental group of larvae (Figure 2). The increase in heart rate from before recovery was significant in 0.25 mM and 0.50 mM PPA groups (P= 0.00088 and 0.0047, respectively), but not significant in the 0.75mM PPA group (P=0.64 and 0.68, respectively).

Morphology

The morphology of the 5 dpf larvae differed between each concentration group (Figure 3). Larvae incubated in 0.25 mM PPA do not appear to differ morphologically from control larvae. However, all larvae at 0.50 mM PPA had a deflated or absent gas bladder. Additional deformities were observed in larvae incubated in 0.75 mM PPA. There was edema of the heart, liver, as well as the intestinal tract region. Additionally, kinking upward of the tail fin was observed along with a curved tail and trunk region.

Discussion

Concentrations of 0 mM and 0.50 mM PPA did not cause fatality after 5 dpf. However, 0.25 mM PPA resulted in 5% fatality at 5 dpf which may have been due to other complications in the development of the larvae which may be due to inbreeding and/or mutation. Larvae incubated in 0.75 mM PPA experienced 15% fatality while 1.0 mM and 1.5 mM PPA was fatal by 5 dpf.

Analysis of the affect of PPA on the lateral line system determined that the reaction time data was inconclusive (Figure 1). There was a significant difference in reaction time between the control larvae and 0.25 mM and 0.75 mM PPA larvae. However, there was no significant difference between control and 0.5 mM PPA. Interestingly, larvae in 0.75 mM PPA were alive, yet they were nonresponsive to the bead drop assay. This may be due to that concentration of PPA causing a toxic effect on the larvae that wasn't harmful enough to kill them, yet disturbed their ability to react to the stimulus.

It would be expected that if the reaction times of each group were consistent, r^2 values would be about 0.95 or above. Regression analysis results were all below 0.60 which leads to the

conclusion that the recorded reaction times were variable. These variable and inconclusive results could be due to error when attempting to drop the bead at the same time for each larva.

As the reaction assays were being performed, a difference in heart rate between each concentration group was observed (Figure 2). Heart rate is important in assessing cardiac function. Any variations from natural heart rate of zebrafish larvae can allude to effects of heart conditions (Luca et al. 2014). Larvae incubated in concentrations of 0.50 mM and 0.75mM PPA had significantly slower heart rates than control larvae. The decrease in the heart rate after incubation in PPA suggests that there was an effect on the cardiovascular system. This is consistent with research done by Claseys et al. (2011) in which it was determined that heart rate decreased in dogs after treatment with PPA. This research can be used in addition to the research done by Galle (2013) to see if there is a connection between the affect of PPA on the touch response and the affect on the cardiovascular system.

Increase in heart rate after recovery was only significant for larvae incubated in 0.25 mM and 0.50 mM solutions (Figure 2). This increase brought their heart rate back to a heart rate that was not significantly different from the control. This leads to the possibility that the larvae treated with these concentrations were able to recover after an overnight incubation in 1X egg water. If their hearts were temporarily damaged, this may be consistent with research done by Poss et al. (2002) in which they found that zebrafish are able to regenerate their hearts by proliferation of cardiomycytes. If this recovery is indeed possible, this would mean that the effect on the cardiovascular system was temporary and did not affect its development. The heart rate of larvae treated with 0.75 mM PPA did not experience a significant increase in heart rate which may be due 0.75 mM PPA causing damage to the development of the larvae. The lack of

recovery may allude to permanent developmental damage on the cardiovascular system. In future research, it may be interesting to allow a longer recovery time in 1X egg water, such as five days instead of only one day, to determine if the larvae treated with 0.75 mM PPA can recover given a longer period of time.

The physical appearance of the larvae differed between concentration groups as well (Figure 3). The gas bladder of 0.50 mM and 0.75 mM PPA larvae was absent. Also larvae exposed to 0.75 mM PPA had various phenotypes such as edema and kinking or curving of the tail and trunk. Over the course of the treatment, the morphological effects became increasingly prominent.

Although the reaction time data was inconclusive, other effects of PPA on the larvae were observed such as changes in heart rate and morphology. In future studies, using a more accurate assay that eliminates human error may produce more significant results. For example, it would be necessary to determine a mechanism in which the time between stimulus and response can be accurately measured. More observations may be necessary to receive more conclusive and significant results for reaction assays. Additional research on the morphological effects of PPA should be done to learn more about the physical changes observed in the larvae. Further research could be done to determine the exact concentration at which the toxicity of PPA becomes high enough to cause the larvae to lose the ability to react. According to my observations, this may be between 0.50 mM and 0.75 mM PPA. There were interesting differences between larvae incubated in 0.50 mM and 0.75 mM PPA. Not only was there a difference of reaction time at 0.50 mM PPA and no reaction at 0.75mM PPA, but the larvae treated with 0.50 mM PPA were able to recover while the larvae treated with 0.75 mM PPA did not recover. The difference in

morphology was also visible in which 0.75 mM PPA larvae had considerably more edema than 0.50 mM PPA larvae. Since PPA has continued use in veterinary science and can still be found in some pharmaceutical preparations across the world, it is necessary to continue studying the possible negative effects that PPA can have on various systems of an organism.

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Table 1. An examination of survival rate of larvae incubated in various concentrations of phenylpropanolamine at 5 days post fertilization.

	Control	**0.25 mM**	**0.50 mM**	**0.75 mM**	**1.00 mM**	**1.50 mM**
Percent Survival (%)	100	95	100	85	0	0

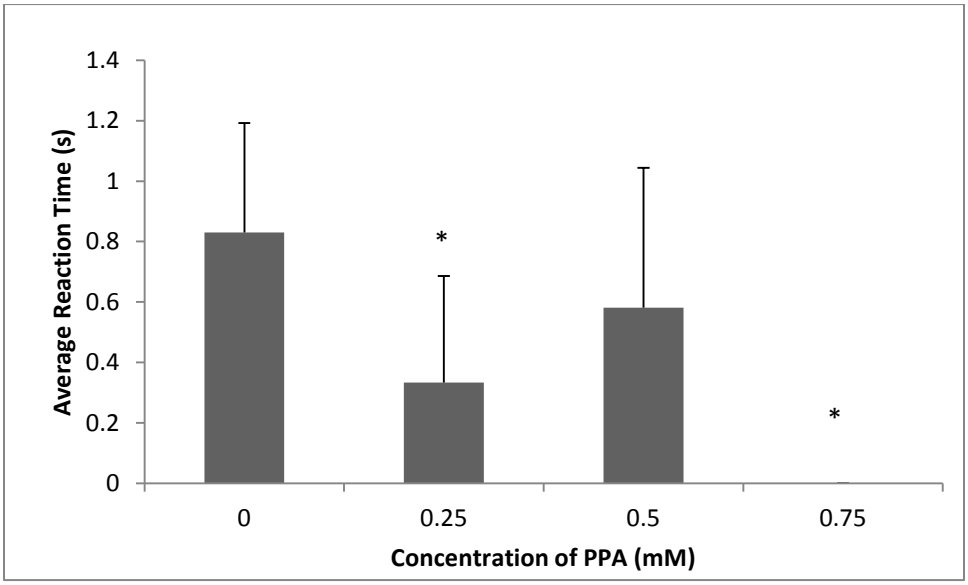

Figure 1. Average reaction time from response assay at 5 days post fertilization zebrafish larvae incubated at various concentrations of phenylpropanolamine (PPA). Means with asterisk (*) are significantly different from the control group. Error bars represent standard error. N=20.

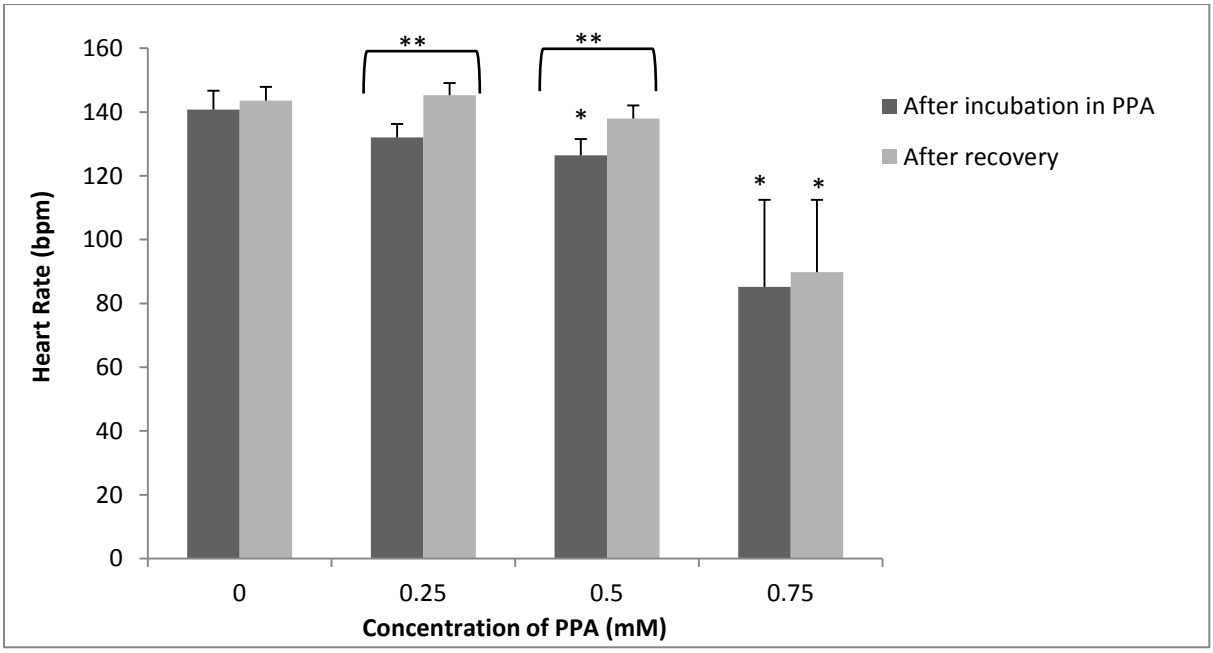

Figure 2. Heart rate of zebrafish larvae after incubation in various concentrations of phenylpropanolamine (PPA). Dark bars represent heart rate in beats per minute after incubation in PPA until 5 days post fertilization. Light bars represent heart rate after incubation in PPA until 5 dpf with subsequent overnight recovery in 1X egg water. Means with asterisk (*) are significantly different from control heart rate. Means with double asterisk (**) represent a significant increase in heart rate after recovery. Error bars represent standard error. N=5.

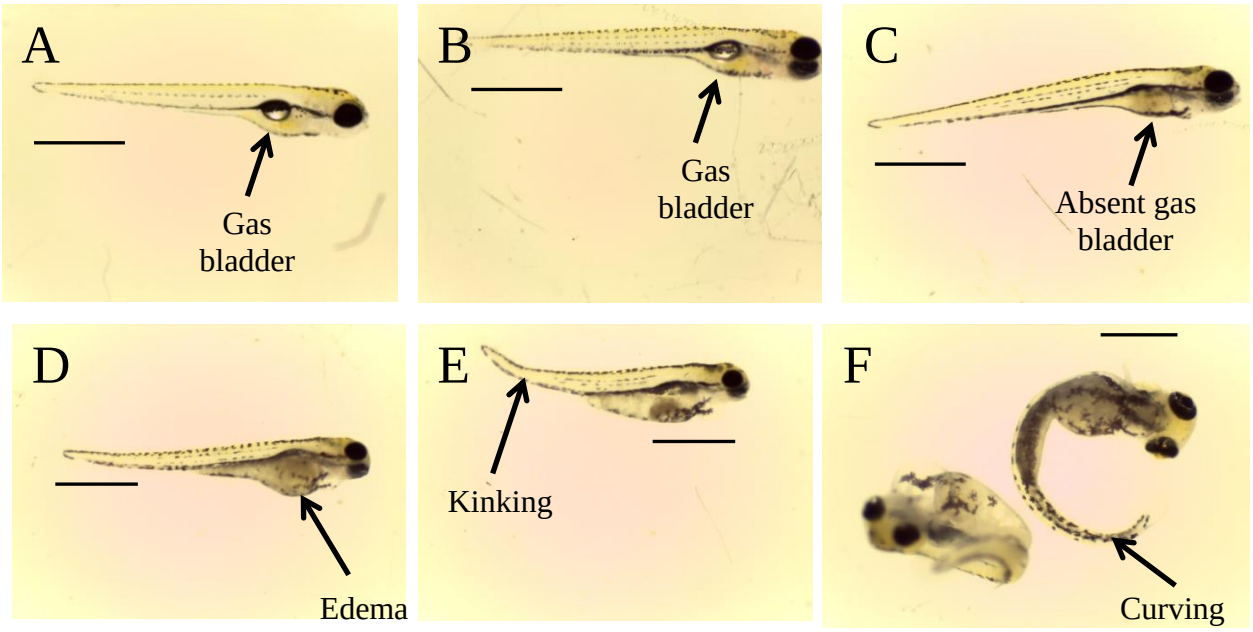

Figure 3. An examination of morphology of 5 days post fertilization zebrafish larvae treated with phenylpropanolamine (PPA). (A) Control larva incubated in 1X egg water. (B) Larva incubated in 0.25mM PPA. (C) Larva incubated in 0.50 mM PPA. (D-F) Larvae incubated in 0.75 mM PPA. Scale bar, 1 mm.