Development of Retinal Degenerative Model Using Lead Acetate (PbAc) in Zebrafish: Morphological and Behavioral Aspects

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Research Article

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Abstract

Lead intoxication reduces cGMP phosphodiesterase activity and enhances the influx of Ca^{2+} in photoreceptor cells that trigger ATP loss. Thus, releasing proapoptotic factors and activating caspase cascade results in retinal cell apoptosis. In the current study, lead acetate (PbAc) induced retinal degenerative model which mimics ocular degenerative disorders like macular degeneration and retinitis pigmentosa. Qualitative and quantitative analysis of retinal histology, with dose and time response parameters, showed 0.08mg/L concentration for 15 days of treatment was the appropriate dose to develop the retinal degeneration model. Its intoxication affected the photoreceptor cells thus contributing to above mentioned disorders. At an ultrastructural level, it was observed that PbAc induce retinal degeneration by damaging outer and inner segments of the photoreceptors especially the rod cells. Escape response behavior also showed a significant decrease in visual response to changing contrasts. Thus, PbAc was inducing degeneration of retina between pigmented epithelium and photoreceptor layer for further retinal regenerative studies. Lead intoxication reduces cGMP phosphodiesterase activity and enhances the influx of Ca^{2+} in photoreceptor cells that trigger ATP loss. Thus, releasing proapoptotic factors and activating caspase cascade results in retinal cell apoptosis. In the current study, lead acetate (PbAc) induced retinal degenerative model which mimics ocular degenerative disorders like macular degeneration and retinitis pigmentosa. Qualitative and quantitative analysis of retinal histology, with dose and time response parameters, showed 0.08mg/L concentration for 15 days of treatment was the appropriate dose to develop the retinal degeneration model. Its intoxication affected the photoreceptor cells thus contributing to above mentioned disorders. At an ultrastructural level, it was observed that PbAc induce retinal degeneration by damaging outer and inner segments of the photoreceptors especially the rod cells. Escape response behavior also showed a significant decrease in visual response to changing contrasts. Thus, PbAc was inducing degeneration of retina between pigmented epithelium and photoreceptor layer for further retinal regenerative studies.

1. Introduction

Zebrafish is a popular research model in vision research due to similarity of its visual system with other vertebrates. Furthermore, it has the ability to regenerate lesioned retina (Gemberling et al. 2013). Müller cells, which are present in INL, are responsible for retinal regeneration in zebrafish. After retinal degeneration, Müller cells become activated and differentiate into various retinal cell types. The ciliary marginal zone is another source of new cells in the retina of adult zebrafish. This source maintains the constant density of rod photoreceptors in the continuously growing zebrafish eye (Brockerhoff and Fadool 2011). These two sources impart lifelong retinal growth and neurogenesis, which is essential to maintain rod density in an ever growing eye (Otteson and Hitchcock 2003; Hochmann et al. 2012). Various animal models of retinal degeneration are available to study the molecular mechanisms underlying this regenerative capacity, allowing for a better understanding of the disease and the development of therapeutic strategies. Hitherto, chemical treatment with retinotoxic substances such as MNU in zebrafish & rodents (Tappeiner et al. 2013; Zulliger et al. 2011; Tsubura et al. 2010) and $CoCl_2$ in

rodents (Hara et al. 2007), excessive light illumination in rodents (Wenzel 2005) and genetic manipulations (Hawes et al. 2000; Reme 1998) have been used to form retinal degeneration models. In this study, environmental toxicant lead is used to induce retinal degeneration in zebrafish. It has been reported that acute lead exposure causes deterioration of rod and bipolar cells resulting in scotopic vision (Fox and Sillman 1979; Fox et al. 1998). Lead poisoning caused swelling of the retinal pigment epithelium in rabbits leading to degeneration of the photoreceptors (Hughes and Coogan 1974; Brown 1974). It is also reported that lead exposure effect cGMP phosphodiesterase activity, enhanced intracellular Ca^{2+} concentration that further leads to increased permeability of the inner mitochondrial membrane. Increased permeability enhances ATP loss, the release of proapoptotic factors into the cytosol, activation of caspase cascade, and chromatin fragmentation that results in photoreceptor degeneration. Rod cell loss is a characteristic feature of aging and AMD, reflecting a continuum from aging changes to the disease. The aim of this study is therefore to develop a retinal degenerative model in zebrafish with PbAc. Histological and behavioral parameters were used to assess the optimum dose to cause photoreceptor degeneration. This study on retinal degeneration induced with heavy metal will not only help to study age related retinal disorder but will cover the environmental aspect too. Further, it will help in understanding the pathways involved in retinal degeneration post exposure to environmental hazardous chemicals and development of therapeutic strategies to treat retinal disorders.

2. Experimental Procedure

2.1 Materials and Methods

Adult wild type zebrafish (*Danio rerio*) (6-12 months old) was habituated to the laboratory conditions for 14 days and housed in un-chlorinated aquarium water at temperature of 28 ± 2°C with constant filtration and aeration. Animals were kept on 14:10 hour light/dark cycle and fed twice a day with aquarium food. All the animals were housed and cared in accordance with the ‘Guide for the Care and Use of Experimental Animals’ approved (PU/45/99/CPCSEA/IAEC/2017/33) by Institutional Animals Ethics Committee, Panjab University, Chandigarh.

2.2 Experimental design

2.2.1 Lead exposure regimen

Lead acetate induced retinal degeneration model was prepared using dose and time response study. 30 mM stock solution of lead acetate was prepared. Four doses of lead acetate, 0.04, 0.06, 0.08 and 0.1 mg/L were used for the dose-response study. Fish was exposed to lead through rearing water. Six eyes per group were enucleated after 3 weeks treatment. Further, time response analysis was done by extracting the whole eye after 2 and 4 weeks of treatment with lead acetate. Eight eyes from each group were used for the experiment. To describe the morphological changes in retinal layers, hematoxylin and eosin (HE) staining was used, and a suitable dose of lead acetate degenerating photoreceptor cells was measured.

2.2.2 Selection of optimum effective dose for regeneration studies

The microscopic examination of ach retina was done and scored semi-quantitatively as follows: no changes, 0; least morphological changes of the ONL in retina, 1; atrophy of ONL, 2; disappearance of ONL, 3; and damage of total retinal layers, 4. The mean score of each group was termed as retinal damage index (RDI). Furthermore, photoreceptor damage rate (PDR) and total damage rate (TDR) was calculated. The photoreceptor damaged area, total damaged area and total area of retina was measured using Image J software.

$$PDR = \frac{Retinal\ area\ showing\ photoreceptor\ degeneration}{Total\ retinal\ area}$$

$$TDR = \frac{Retinal\ area\ showing\ the\ total\ damage}{Total\ retinal\ area}$$

RDI, PDR and TDR were calculated for dose response analysis and RDI was calculated for time response analysis. Concentration, at which atrophy of ONL was observed, was taken as an optimum dose to produce retinal degeneration model.

2.3 Histological processing

Zebrafish was euthanized using 0.04% tricaine methanesulphonate. Eyes were extracted and fixed overnight in 10% phosphate buffer formalin. The tissue processing was done following the method of Pearse (1968). Then tissue was embedded in paraffin wax and cut into 5 µm thick sections for histological analysis. The thickness of the outer nuclear layer, the inner nuclear layer and the photoreceptor damage were measured using Image J software.

2.4 Ultrastructural investigation

Sample preparation

Eye was extracted and placed in 5% glutraldehyde at 4˚ C for overnight. Next day retina was extracted and washed with 0.1 M Sorenson's buffer. Post fixation was done with 1% osmium tetraoxide at 4˚C for 1 hr. The tissue was subjected to dehydration with ascending series of acetone upto 100% concentration at 4˚C. This retinal tissue was further treated with different ratios of acetone and amyl acetate (Acetone:Amyl acetate- 3:1, 1:1, 1:3) at 4˚C. Finally, tissue was dried performing CPD. Dried sample were loaded on metallic specimen stubs and specimens were coated with platinum ion using Hitachi sputter coater. The conducted specimen was examined under Field Emission Scanning Electron Microscope (Hitachi SU1080, Japan) at CIL, Panjab University, Chandigarh.

2.5 Visual behavior analysis

Visual sensitive behavioral analysis was done according to the method of Li and Dowling (1997). This method allows to measure behavioral thresholds of cones and rods as well as the time of photoreceptor adaptation. The experimental apparatus consists of a circular plastic container surrounded by a rotating drum. A white light source is used to illuminate the drum from above. Zebrafish swim slowly in circles when confined in a circular container (10 rpm). However, when confronted by a black segment rotating outside the container displayed a robust escape response. To evaluate quantitatively the behavioral responses, the ratio of the number of escape responses to the total number of encounters between the fish and the rotating segment was measured. The video was recorded using Nikon camera and number of encounters with black bar and escape was recorded in different groups to check the visual acuity of zebrafish.

$$Escape\ behaviour = \frac{Number\ of\ escape\ responses}{Number\ of\ encounters\ between\ fish\ and\ rotating\ segment}$$

2.6 Statistical analysis

All the values were expressed as mean ± S.E. for all the experiments. Retinal layers thickness data were analyzed by one-way ANOVA followed by a post-hoc Tukey's test. Non parametric test were done for behavior analysis followed by the Kruskal-wallis test. All statistical analyses and data were accomplished using IBM SPPS 21 software. Value with p £ 0.05 was considered statistically significant.

3. Results

3.1 Histological evaluation of retinal degeneration

Dose response is a framework in which hazardous chemical assessment is done and from which environmental regulations are derived (Calabrese 2014). In this study, the changes in photoreceptor layer were assessed in dose and time dependant manner.

3.1.1 Dose response analysis

Histopathological images depicted regular shaped retina of the control group and layers were typically organized (Fig.1). The thickness of plexiform layers and thickness of ONL was decreased in 0.06 and 0.08 mg/L treated retina respectively (Fig.1A, B). ONL was damaged under these conditions whereas fewer changes were observed in inner nuclear layer and ganglion cell layer (Fig.1). Vacuolation in the photoreceptor layer was observed in 0.08mg/L treated group that show photoreceptor cell damage (Fig.1C). At the highest concentration of 0.1 mg/L, substantial damage in the plexiform layer and ONL has observed that show deterioration of photoreceptor cells and synaptic junctions. Quantitative assessment of retinal layer thickness depicted dose dependant decrease in thickness of ONL, INL and photoreceptor layers (Fig.2A). Highest RDI was calculated at 0.1 mg/L concentration that showed damage in all retinal layers. PDR increased gradually in dose dependant manner and peaked at 0.08

mg/L and TDR was maximum at 0.1 mg/L (Fig.2B). Appropriate concentration to induce selective photoreceptor degeneration is therefore calculated to be 0.08mg/L.

Minimum changes were observed in all retinal layers including photoreceptor layer at 0.04 and 0.06 mg/L concentration. The atrophy of ONL was expressed at the concentration of 0.08 mg/L and it was scored 2 of RDI. PDR and TDR were calculated. Optimum damage in photoreceptor was observed at concentration of 0.08 mg/L and highest TDR was observed at 0.1 mg/L (Fig. 1 D) of lead acetate.

3.1.2 Time course analysis

The histopathological results of time course analysis for zebrafish retina treated with 0.08 and 0.1 mg/L of lead acetate are presented in Fig.3. RDI value was 2 at 0.08mg/L and more than 2 at 0.1 mg/L which depicted atrophy of ONL (Fig.4). More severe lesions including damage in ONL, INL, and GCL were seen after 30 days of treatment at both 0.08 and 0.1 mg/L. Thus 0.08mg/L can be taken as the optimum dose to induce retinal degeneration in zebrafish.

3.2 Ultrastructural analysis

FESEM showed a seven-layered structure of the retina depicting the topography of various retinal cells (Fig.5A). Under the electron microscope, the zebrafish retina showed rod and cone photoreceptors. Rods are long and slender in shape. Connecting cilium connects outer and inner segments of the photoreceptor (Fig.5B). The outer and inner segments of rods are thinner than the cones. The outer segments of the rod contain visual pigment rhodopsin in bound disks of the membrane. The electron microscopy study found that the cones had a conical outer segment, a cylindrical ellipsoid, and a rounded inner paraboloid segment (Fig.5C). Irregularity in the photoreceptor layer was observed in the retina treated with lead (Fig.5D). In degenerative conditions, the outer segment disc membrane appeared to be vesiculated prior to deterioration of the outer segment. Loss of discs was observed in lead induced degenerative model. The rod synaptic terminal withdraws its position from OPL (Fig.5E).

3.3 Behavior analysis

The escape response from the black bar in the 0.04mg/L treated group was similar to the control group (Fig.6A). Escape from the black bar significantly reduced with an increasing concentration that depicted reduced visual activity in zebrafish eyes. This study showed that initiation of visual startle response depends on the ability to detect contrast. Change in the sensitivity to contrast and alteration in the behavior was observed with increasing concentration in this study (Fig.6B-E). Similar observations were reported in zebrafish when developmentally exposed to lead (Rice et al. 2011; Xu et al. 2016). In this study, visual responses decreased significantly with an increase in the concentration of lead.

4. Discussion

The key cause contributing to vision loss is photoreceptor degeneration. One such retinal degenerative disease is retinitis pigmentosa (Bhatti 2006; Hartong et al. 2006). To understand the pathomechanisms

lying behind it, various animal models have been created. MNU-treated of rodent and zebrafish models are a common example of this. It shows typical rod degeneration after treatment with MNU. In contrast, ouabain and light treatment models showed the damage to the whole retina (Fimbel et al. 2007; Sherpa et al. 2008; Battista et al. 2009; Thomas et al. 2012). The collation of these degeneration models helps to decipher the specific molecular pathways that are involved in photoreceptor degeneration. Fox et al. (1998) observed the selective loss of retinal photoreceptors after treatment with lead in rodents. He et al. (2000) also analysed the opening of permeability trasition pore of mitochondria that resulted into selective rod photoreceptor apoptosis in rodents. In a case study, loss of vision was also observed in humans after acute exposure to lead which affected rod mediated electroretinography (Gilhotra et al. 2007). Chen et al. (2012) has observed the clear phenotypic and behavioral effects in zebrafish following treatment with 0.06 mg/L of lead. Thus the retinal degenerative model was prepared using PbAc in zebrafish which is a popular model because of its persistent retinal neurogenesis and retinal regeneration after severe harm (Johns and Fernald 1981; Yurco and Cameron 2005; Sherpa et al. 2008). Analysis of retinal regeneration mechanisms can help recognize cells and pathways with regeneration potential as well as allowing researchers to compare zebrafish to other organisms that do not show regeneration (Tappeiner et al. 2013).

In this study, sequence of retinal changes was observed post treatment with PbAc. In dose response study, highest dose of PbAc (i.e. 0.1mg/L) disrupted all the retinal layers. Changes in photoreceptor layer were observed at 0.08mg/L concentration. After treatment with 0.08 mg/L of PbAc, histological analysis showed marked cell reduction and vacoulation in ONL, suggesting photoreceptor loss. Fish treated with 0.1 mg/L for 2 weeks demonstrated detachment of INL from OPL and decreased thickness of the plexiform layer. Treatment with 0.08 and 0.1 mg/L for 4 weeks showed disruption in the organization of all retinal layers including outer and inner nucleated, plexiform layers and ganglion cell layer. Ultrastructural investigation revealed the degeneration of cones and rods in lead treated group as compared to control. Compared to cones, the withdrawal of rod internal segments from OPL was more pronounced. Fox et al. (1994) revealed the effect of lead intoxication on rod cells in their previous studies. This is due to rod specific cGMP phosphodiesterase inhibition by lead. It results in inhibition of cGMP hydrolysis and enhanced concentration of cGMP which causes increase in calcium content (Fox et al., 1994). Calcium ions play a role in growth, differentiation and synaptic activity under normal physiological conditions. While an increase in intracellular Ca^{2+} concentration is needed for proper cell function, too much influx and release of Ca^{2+} ions from the intercellular space can cause cell death. Pb accumulation in the body leads to the damage of mitochondria which mainly regulate intracellular calcium concentration. The augmentation of electron transport chain in mitochondria by increased influx of calcium leads to the production of reactive oxygen species (ROS). Studies have suggested that a long-term elevated intracellular concentration of Ca^{2+} plays a fundamental role in rod apoptosis (Nicotera and Orrenius 1998; He et al. 2000). Elevated calcium levels result in excessive energy expenditure and decreased energy production (Needleman et al. 2002). Persistently elevated levels of Ca^{2+} in the mitochondrial matrix induce the apoptosis effector phase accompanied by the irreversible opening of mitochondrial ion channels (He et al. 2000; Fox et al. 2003). As a consequence, mitochondrial membrane

depolarization, cytochrome c release, activation of caspase, and chromatin fragmentation occurs. After mitochondrial function is compromised, glutamate's mild synaptic transmission becomes pathological excitotoxicity, resulting in cell death. (Sanders et al. 2009).

Further visual behavioral analysis showed change in sensitivity to the contrasts and alteration in visual response which helped to determine the impact of toxicant. Visual response to black bar significantly degraded in increasing dose dependant manner. Escape response was decreased when fish was treated with 0.08mg/L of PbAc. Highest concentration of lead acetate i.e. 0.1 mg/L augmented the erratic behavior in zebrafish. Thus 0.08 mg/ L concentration showed to disrupt the visual efficacy. Similar behavioral deficits such as visual startle response were observed in zebrafish larvae when exposed to 30 nM lead. A shift in the sensory response indicates that the functionality of either the ON or OFF center receptive fields has been compromised (Rice et al. 2011). In addition, interference with the integration of information may occur in higher brain centers as Pb^{2+} reduces the level of dopamine in the optic tectum of rainbow trout (Rademacher et al. 2001). This suggests that zebrafish are an appropriate model for lead induced sensory-motor deficits.

Retinal morphology observed after 15 days of treatment with lead acetate resembles the MNU induced rod cell degenerative model of zebrafish. This suggests that similar cytological processes are involved in both genetic and metabolic aspects of photoreceptor cell degeneration. Apoptosis is actually the final typical pathway and succeeds the morphological changes of photoreceptor cells in many instances. In this study, lead induced retinal degeneration mimics the retinal photoreceptor degenerative model and it will help in understanding the etiology and vulnerability of photoreceptors to heavy metals in the environment.

5. Conclusion

Zebrafish is a versatile tool to study retinal degeneration and regeneration pathways. In this study, zebrafish was treated with PbAc to induce retinal degeneration. The assessment of histological and behavioral parameters showed that 0.08mg/L of lead acetate is optimum to induce photoreceptor degeneration. It can consequently be hypothesized that 0.08mg/L of lead acetate can be used to prepare a novel retinal degeneration model of zebrafish. This retinal degenerative model can help in understanding the pathways involved in retinal degeneration post exposure to environmental hazardous chemicals. Understanding of these pathways will not only lead to the development of therapeutic strategies to treat retinal disorders but also help in heavy metal risk assessment studies pertinent to vision at the environmental level.

Declarations

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Conflicts of interest

There are no conflicts of interest to declare.

Availability of data and material – Not applicable

Code availability – Not applicable

Author's Contribution

R.K. and V.G. equally contributed in research design, experimental work, drafting of manuscript and final analysis of manuscript.

Ethics approval

All the animals were housed and cared in accordance with the 'Guide for the Care and Use of Experimental Animals' approved (PU/45/99/CPCSEA/IAEC/2017/33) by Institutional Animals Ethics Committee, Panjab University, Chandigarh.

Consent to participate – Not applicable

Consent for publication – Not applicable

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Figures

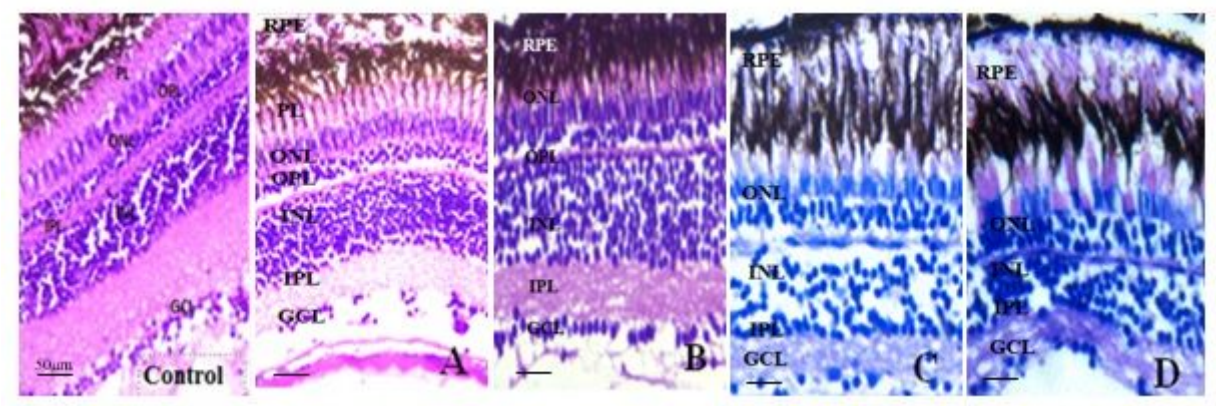

Figure 1

H&E staining of zebrafish retina treated with four different doses of lead acetate for 21 days A) 0.04 mg/L: depicted minor changes in ONL B) 0.06 mg/L: changes in ONL with photoreceptor layer degeneration and changes in INL C) 0.08 mg/L: changes in ONL, INL and OPL and IPL D) 0.1 mg/L: disrupted RPE, vacuolation in ONL, damaged photoreceptor layer (400X). Abv. ONL: outer nuclear layer; INL: inner nuclear layer; OPL: outer plexiform layer; IPL: inner plexiform layer; GCL: ganglion cell layer; PL: photoreceptor layer

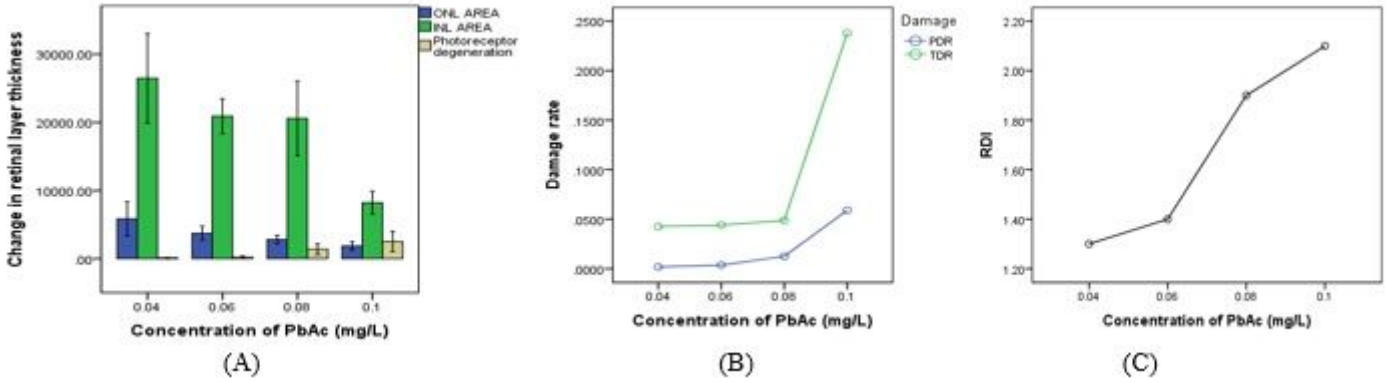

Figure 2

Histological assessment of the retina (A) Thickness of retinal layers ONL, INL and photoreceptor layer decreased in increasing dose dependant manner (B) Photoreceptor damage rate (PDR), Total retinal damage (TDR) and (C) Retinal damage index (RDI) after 21 days of treatment with lead acetate

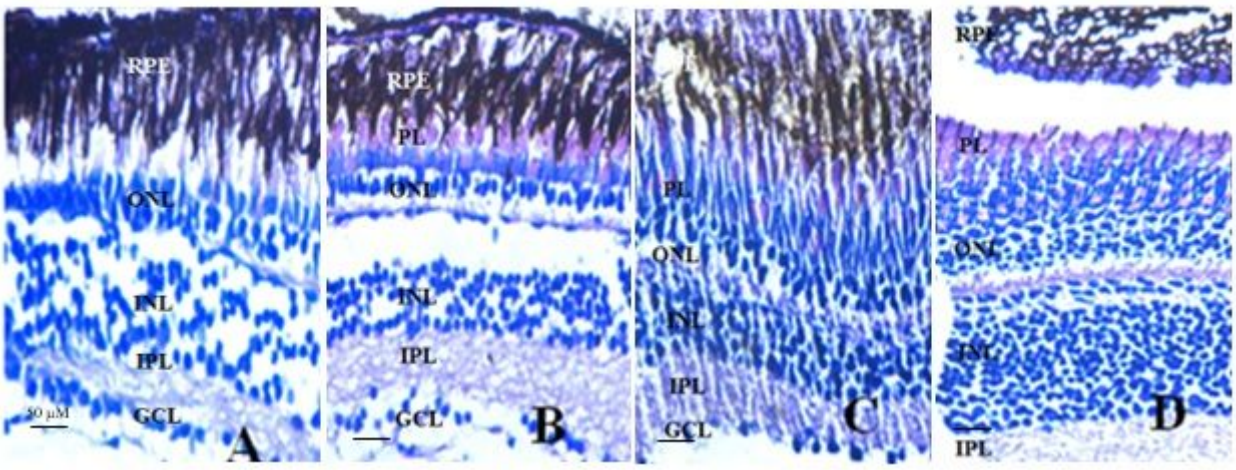

Figure 3

H&E staining of zebrafish retina treated with lead acetate (0.08 and 0.1 mg/L) for 15 (A-B) and 30 (C-D) days at (400X) A) Vacuolation in outer nuclear layer, damaged photoreceptor layer B) Changes in the outer nuclear layer and inner nuclear layer C) Damage in the outer nuclear layer, plexiform layers and the inner nuclear layer D) Delamination of the outer nuclear layer, damage in the inner nuclear layer and plexiform layers. Abv. ONL- Outer nuclear layer; INL- Inner nuclear layer; OPL- Outer plexiform layer, IPL- Inner plexiform layer; GCL: Ganglion cell layer; PL: Photoreceptor layer

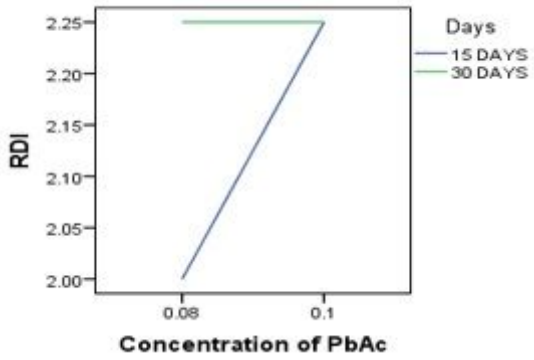

Figure 4

Graph depicting retinal damage index (RDI) after 15 and 30 days of zebrafish treatment with lead acetate

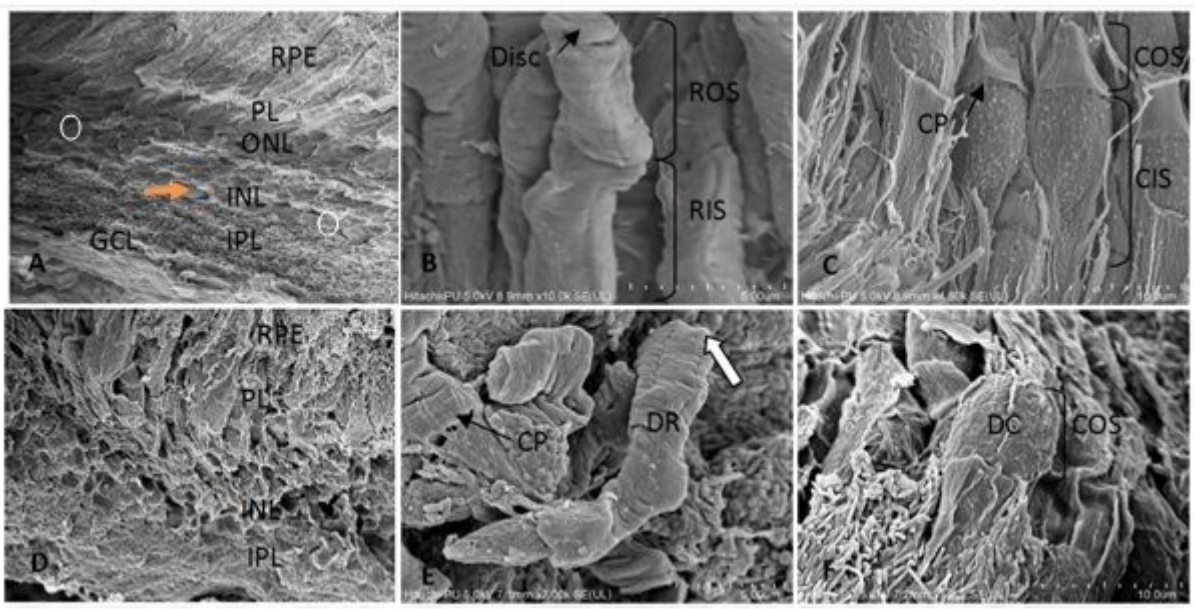

Figure 5

Ultramicrophotographs of Control and treated retina (A-C) Control A) Intact retinal layers including RPE, PL, ONL, OPL, INL, IPL INL depicting bipolar cells and horizontal cells (circle), Müller cell in INL (arrow) and synaptic bodies in IPL B) Normal rod cell with intact IS and OS C) Cones with OS and IS joined with connecting piece (D-F) Degenerative model D) Rods depicting OS and IS with damaged discs E) Rod photoreceptor depicting outer and inner segment with connecting piece F) Damaged COS and CIS Abv. ONL- outer nuclear layer, INL- inner nuclear layer, OP- Outer plexiform layer, IPL- Inner plexiform layer, GCL- Ganglion cell layer; PL-Photoreceptor layer, DR-Damaged rod, CP-Connecting piece, ROS-Rod outer segment, RIS-Rod inner segment, COS-Cone outer segment, CIS-Cone inner segment, RPE-Retinal pigmented epithelium

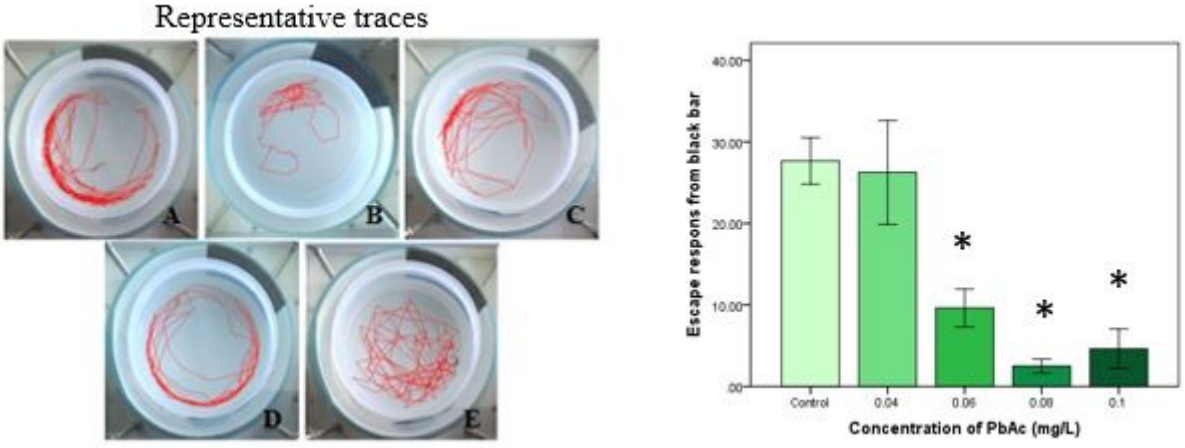

Figure 6

Visual acuity- Automated behavioral characterization by video tracking software and quantitative measurement of escape response from black bar in adult zebrafish after treatment with lead acetate. A) Control showing escape from black bar B) 0.04mg/L- showing decreased escape response C) 0.06mg/L-

decreased escape behavior D) 0.08 mg/L- minimal escape response from black bar E) 0.1mg/L- depicting erratic behavior Data represented as Mean±S.E. using Kruskal-Wallis test $p<0.05$

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GraphicalAbstract.jpg