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In ovo exposure of F-ions and organo-fluoride insecticide (Bifenthrin) cause developmental anomalies of eye in chick embryos

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ABSTRACT

Objective: The developmental abnormalities of the in-ovo exposure of Fluoride ions (F-ions) and Bifenthrin (BF) on the embryonic chick eye were investigated.

Materials and methods: 165 fresh fertilized eggs of zero day and 40–50 g weight were divided into three groups (55 eggs each) on the basis of inter-vitelline treatment of eggs on zero day of study: 1) Control group (CG); 0.1 ml of 5 % DMSO aqueous solution 2),3) Fluoride group (FG), and Bifenthrin group (BFG); 0.01 mg/kg F-ions (from NaF) and 0.01 mg/kg BF in 0.1 ml of 5 % DMSO aqueous solution respectively. After incubation for 14 days at 37 ± 0.5 °C embryos were externalized. Eyes of each embryo were removed for micro-anatomical, micrometric and histopathological studies.

Results: The histological sections have shown denser and enlarged marginal mitotic region of the developing eye lenses in FG and BFG. In vertical sections of the eye lenses the nuclei of the crystalline cells in FG and BFG show a highly depressed bow shaped arrangement. Moreover, the nuclei of the core crystalline cells of the lens were apparently smaller in FG and BFG than CG. Out of the six anatomical layers of the retina the nuclear and the plexiform layers were highly enlarged in FG and BFG, similarly the three corneal cell layers (endothelial, parenchymal and epithelial) were enlarged in FG and BFG than CG. The morphometric, histometric and micrometric estimations also show significant variations in FG and BFG than CG.

Conclusion: The results indicate subtle developmental anomalies of the eyes attributable to the F-ions and BF exposure indicating their developmental neuro-optic disruption potentials. Results further revealed higher toxicity of BF as compared to F-ions.

1. Introduction

Fluoride has been frequently used in water purification systems and toothpastes. The organo-fluoridated insecticides (OFI) like Cyhalothrin and BF has been preferred over organophosphate group and are still being used frequently in work places as well as agricultural and domestic sectors presuming their rapid biodegradation and thus non-accumulative and least toxic nature for the non-target animals and humans without any authentic studies upon their metabolism, pharmacokinetic and pharmacodynamics [1,2]. Unfortunately, both inorganic and organic (BF) forms of F have been reported to induce various toxicological manifestations even at a low dose of exposure in animals [3,4]. It is a potent environmental toxicant, having potential to cause dental fluorosis, skeletal fluorosis and toxic effects on different body organs, such as brain, liver, kidney and spinal cord [5,6]. Chronic F exposure has been reported to induce oxidative stress, enhance lipid peroxidation, mitochondrial damage and apoptosis in various body tissues and organs [4,7]. High dose exposure of F has been found to be toxic to retina. It can cause a widening of retinal vessels leading to retinitis and involvement in cataract [8].

Vertebrate eye is a complex organ that develops through intricate molecular and cellular processes utilizing both the differentiating skin

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and neuro-ectoderms [9,10] and thus can be used as an indicator of developmental neuro-ectodermal toxicity. Chick (Gallus domesticus) is an excellent model of experimental vertebrate embryonic developmental studies. System, having large size of the embryonic eye, being extensively utilized for studying the development and growth of the eye [11], for the collection of larger amounts of tissue for cell culture and molecular analysis. Additionally embryonic chicks can regenerate their retinas at specific stages, making them also a very good model for regenerative eye biology [11]. In-ovo BF and F-ions exposure in the developing chick embryo resulted in neuro-developmental abnormalities [12]. However, there exists no previous information that may highlight the toxicity of F-ions and/or fluoridated organics (FOs) with respect to developmental neuro-ophthalmic development in the avian system. In present study the micro-anatomical and histopathological toxicity of an F-ions and a fluoridated insecticide (Bifenthrin) were investigated in developing chick embryos.

2. Materials and methods

2.1. Ethical approval process

All procedures of this study were approved by the ethical committee of the department of Zoology, University of Sargodha, Pakistan (Code: SU/Zol/312) Dated 16 Feb, 2021.

2.2. Egg collection

165 fresh fertilized eggs of zero day and 40–50 g weight were obtained from commercial supplier. These eggs were divided into three groups (55 eggs each);.

i) control group (CG): Eggs injected with 0.1 ml of 5 % DMSO aqueous solution.
ii) Fluoride group (FG): Eggs injected with 0.1 ml of 0.01 mg/kg solution of NaF in 5 % DMSO.
iii) BF group (BFG): Eggs injected with 0.1 ml of 0.01 mg/kg solution of BF in 5 % DMSO.

2.3. Preparation of NaF and BF solutions

Desired dose (0.01 mg/kg) of NaF and technical grade BF (Batch No# Auc/20130611, manufactured by Be Star China; donated by "Auriga Chemical Enterprises", Lahore Pakistan) were prepared in 5 % DMSO aqueous solution (the single dose volume for each animal remained constant (0.1 ml) whereas the concentration of NaF and BF was adjusted by appropriate dilutions of the stock solution in accordance with the egg weight).

2.4. Dose administration

Eggs shells were cleaned and sterilized with 70 % alcohol. A window is created by a drop of HCl in the center of each egg. All eggs were placed on one side for 5 min, so that embryos rise on the top. The sterilized syringe of 1 ml was used to administer group specific treatment to each egg. The needles were injected horizontally from the window into the yolk sac. Immediately after injection, the hole was sealed with sticking tagging taps labeled with group names. Weights of eggs were measured on digital balance of 0.1 g precision and noted.

2.5. Incubation

A medium sized automatic incubator (Mammert) with digital temperature display and 200eggs capacity was used. The eggs were rotated twice a day. The incubator was maintained at an optimum temperature of 37 ± 0.5 °C. The humidity was maintained at 65 % in incubator.

2.6. Recoveries

On the 14th day of incubation eggshells were cracked from the broader end with the help of forceps, the inclusions were transferred in a china dish partly filled with 0.8 % saline. The embryos were finally removed carefully from the top of the yolk with the help of camel hair brush and forceps. Soon after the embryos were fixed in acidified formyl ethanol for 24 h.

2.7. Dissection

The embryos fixed in formyl ethanol were dissected under a binocular Stereomicroscope (MSC-ST60). The eyes were removed intact from the sockets with help of microsurgical scissors and scalpels and the fine forceps.

2.8. Morphometric measurements of eye

Following morphometric measurements were taken of the eyes:

- Length and width of each eye was measured with the help of a Vernier caliper
- Weight of each eye was obtained on a digital weight balance
- Volume of each eye was measured through water displacement method
- Density of each eye was calculated by employing the formula: D = W/V

2.9. Histological preparations of eyes

The intact eyes balls were dehydrated gradually in various grades of ethanol (20, 30, 40, 50, 60, 70, 80, 90, 95, 100 %) interphases and media changes (alcohol-xyline: 75–25, 50–50, 25–75 % and lastly pure xylene) and embedding (xylene-wax: 75–25, 50–50, 25–75 % and lastly pure wax) so that the organs mays not shrink during this process. The last three steps of embedding (i.e. 50–50, 25–75 % xylene-wax interfaces and pure molten wax) were carried out at 40, 45 and 55 C respectively in a water bath. The embedded organs fixed in rectangular wax blocks to obtain serial transvers Section (3-5 μm thick) on a rotary microtome (ERMA TOKYO 422). The sections were transferred on glass slides for H and E staining and finally for the histological and micrometric studies.

2.10. Histopathological and micrometric studies

Digital photographs of the medial eye sections were captured using camera fitted Trinocular research microscope (Labomed CXR2) on 100 × and 400 × . The snapshots were analyzed for histopathological signs and micrometric estimations through corelDRAW11 software The micrometric data were analyzed statistically by One Way Analysis of Varience (ANOVA) and Tukey's Multiple Range Test (TMRT) through IBM SPSS statstics 2023.

3. Results

3.1. Micro anatomical and histological results

3.1.1. Histology of lens

3.1.1.1. Control group. The TS of eye lenses in CG embryos were ellipsoidal in shape covered exteriorly with a cellular epithelium- cuboidal in the middle and columnar at the margins. Interiorly the crystalline lenses were covered with an opaque non-cellular layer- thicker in the middle and gradually becomes thin towards the margins. The Columnar epithelial margins of the outer cellular layer bend to fuse with the marginal mitotic zone of the developing crystalline lens. Cells of the lens

proper gradually differentiate form marginal mitotic zone to the central thickest crystalline zone. The cellular nuclei of the central zone were placed in the middle and the transparent crystalline cytoplasm extending from anterior to posterior margin all along the girth of the lens. Thus the central zone of the embryonic eye lens appeared transparent. The cellular nuclei of the proper lens arrange themselves in such a way that they form a bow like arrangement in the middle of the lens slightly depressed from the middle then deflecting outwardly in the flanking middle zone and curve inwards at the marginal zone (Fig. 1A).

3.1.1.2. Fluoride group. The TS of the eye lenses in the FG were similar to that of the CG in gross structural layout, however the marginal mitotic zone was much enlarged and the nuclei of the central zone were arranged into a deeply bended bow like structure. The cellular cytoplasm of the middle zone appears less transparent while some internal lesions of this zone were also visible, furthermore the cellular nuclei appeared rounded and smaller as compared to the slightly elliptical and larger cellular nuclei found in the middle zone in control group (Fig. 1B).

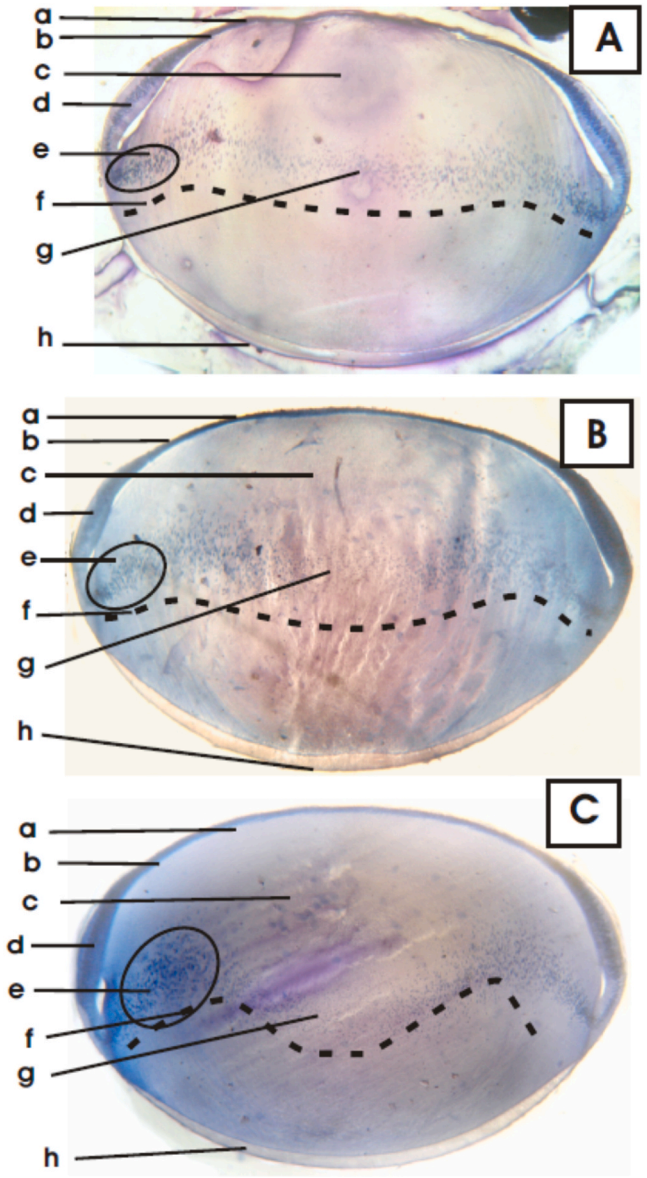

Fig. 1. Hematoxylin and Eosin stained histological sections (100 X) of lens of 14 day chick embryo. **A**:CG, **B**:FG, **C**: BFG (a) anterior capsule, (b) epithelium, (c) cortex, (d) equator, (e) mitotic region, (f) nuclear bow, (g) nucleus, (h) posterior capsule.

3.1.2. Bifenthrin group

The orientation and disposition of lens in BFG was similar to that of CG. However the marginal mitotic zones were denser. The bow like arrangement of the central zone cells nuclei showed even sharper curvatures than that of FG. However, like FG the size and shape of the nuclei of the central zone of the lens were rounded and smaller as compared to the CG where they appeared elliptical and comparatively larger. Similar to FG the middle zone of the lens appeared less transparent (opaque) than that of control (Fig. 1C).

3.1.3. Histology of retina

Six distinct micro-anatomical layers of retinal cells and cellular projections (namely - in the anti-vitreous to vitreous alignment- 1: pigmented epithelium; 2: inner and outer segments of rod and cone cells; 3: nuclear and plexiform layer; 4: bipolar layer; 5: ganglionic layer and; 6: the nerve fiber layer) were clearly identifiable in all the three groups, however the cells in pigmented epithelium in CG were properly aligned into a uniform columnar arrangement as compared to the pseudostratified arrangement found in FG and BFG. The nuclear and plexiform layer was slightly thicker in FG and BFG than the CG. While the retinal stem cells visible in bipolar layer in CG were mostly were absent in FG and BFG. A very well placed and distinctly identifiable nerve fiber layer was visible CG only (Fig. 2).

3.1.4. Histology of cornea

The three distinct micro-anatomical layers of the corneal cells (called epithelium, parenchyma and endothelium) were clearly identified in all the study groups. The corneal TS in BFG was thicker than CG and FG. This difference in thickness was seemingly attributable to the less densely populated parenchyma containing wide inter cellular spaces. The parenchymal epithelium in CG was thin and transparent in contrast to the thick granular and pigmented epithelium of the BFG cornea. The germinal endothelium in BFG also less dense than CG (Fig. 3B1&B3). On the other hand, like CG the cornea in FG was well developed showing well placed endothelium, parenchyma and epithelial cell layers showing no obvious signs of histopathology (Fig. 3B2).

3.2. Morphometric results

3.2.1. Mean weight, volume and density of chick embryo eye

Significant difference ($p \leq 0.05$) among the groups for mean weight, volume and density of intact eye balls of the developing chick embryos was observed. Post hoc analyses show significant ($p \leq 0.05$) decrease in mean weight and density in FG and BFG to that of the CG, contrarily the mean volume of the eye in FG and BFG was significantly ($p \leq 0.05$) higher than CG (Table 1).

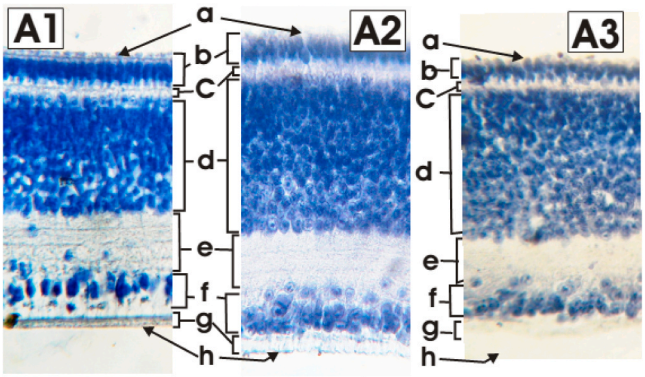

Fig. 2. Hematoxylin and Eosin stained sections (400 X&100 X) of retina of 14 day chick embryo. **A1:** CG, **A2:** FG, **A3:** BFG (a) anti-vitreous side, (b) pigmented epithelium, (c) rod & cone cells layer, (d) nuclear & plexiform layer, (e) bipolar layer, (f) ganglionic layer, (g) nerve fiber layer, (h) vitreous side.

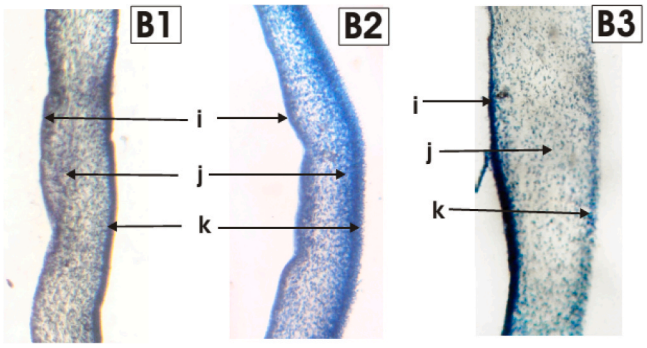

Fig. 3. Hematoxylin and Eosin stained sections (400 X&100 X) of cornea of 14 day chick embryo. **B1:** Cornea of CG, **B2:** Cornea of FG, **B3:** Cornea of BFG (i) endothelium, (j) parenchyma, (k) epithelium.

3.3. Histometric results

3.3.1. Mean breadth of cornea

The mean breadth of cornea of the embryonic eye revealed highly significant difference among the groups ($p \leq 0.0001$), post hoc analyses showed significantly higher ($p \leq 0.05$) mean value for BFG than the CG and FG (Table 1).

3.3.2. Mean length, width of lens

Significance difference ($p \leq 0.05$) was noted both for mean length and width of the eye lens among the groups, post hoc analyses indicate significantly higher mean values in BFG than the FG and CG (Table 1).

3.3.3. Mean breadth of successive retinal layers of chick eye

Statistical Analyses revealed no significant variation among the groups for mean breadth of pigmented epithelium, nuclear and plexiform layer and, the nerve fiber layer. Contrarily significant variations ($p \leq 0.05$) among the groups were noted for mean breadth of bipolar and ganglionic layers. Further post hoc analyses of the data showed significantly lower mean breadth of bipolar layer in BFG than CG whereas the mean breadth value of ganglionic layer was significantly higher in FG than BFG and CG (Table 1).

3.4. Micrometric results

Mean cell density (per 100 μ^2) of embryonic eye lens showed. Post hoc analysis of data for mean length and width of lens using TMRT indicated significant increase ($p \leq 0.05$) in BFG as compared to CG, However FG showed statistically no difference ($p > 0.05$) with either CG or BFG. Post hoc analysis of cell density data indicated significant decrease ($p \leq 0.05$) in FG and BFG as compared to CG (Table 1).

4. Discussion

Animal eye is a fascinating sense organ which develops jointly by the skin and neuroectoderm [13]. The differentiation of eye starts and completes far earlier than many of the other body organs. As it partly develops from the neuro-ectoderm, the differentiation of eye can serve as an excellent model for the study of neuro-toxicological impacts of various environmental chemicals. On the other hand, the development of eye has not been explored for various established neuro-toxicants, thus the results obtained in present study highlights newer insights which are not directly comparable with any such previous studies. The findings suggest that both the F-ions and fluoridated organics (FOs) can potentially bring about various alterations in development of vertebrate eye as indicated by a general increase in eye volume and a simultaneous decrease in eye weight. The differentiation of lens was critically effected as indicated by sharply bended bow like arrangement of the crystalline cells nuclei in FG and BFG. The differentiation of lens completes even faster than that of the retina. At the same time lens is suspended in eye ball between two fluid filled cavities, i.e. aqueous humor towards the cornea and vitreous humor towards the retina. Logically the lens is exposed to the toxicological impacts of F-ions or FOs accumulated in the two fluid filled cavities of the developing chick eye. Fluoride and FOs seems to hamper the differentiation of crystalline region of the lens as indicated by the micro-nuclear formations and the opaque appearance of this portion of the developing eye lenses in FG and BFG. It seems that the lack of differentiation presumably causes cellular necrosis in middle portion of the lens while the marginal areas continue to produce new cells through mitosis. Although there were no overt signs of histopathology in TS of retina, however F and BF exposure seems to interfere with the differentiation of retina as well- thereby causing a general enlargement in nuclear and plexiform layer and the ganglionic cell body layer in FG and BFG with a concurrent decrease in thickness of NFL in FG and poor development of NFL in BFG which indicates neuro-developmental toxicity. Overall the results of this study indicate that F exposure in developing chick at a dose of 0.01 mg/kg may leads to histopathological impacts on the developing eyes and may also affect the development of nervous system as well.

5. Conclusion

Results show that the general impression of non-accumulative and thus safer insecticides for human and pet animals (chick) need a thorough revision; particularly there is a genuine need of investigation from the stand point of the persistent toxicity of halogens and particularly fluoridated pyrethroids such as BF and Lambda- cyhalothrin. This pilot

Table 1
Variations in morphometric, histometric and micrometric readings of Eye of developing chick embryo.

Sr No	Histometric and micrometric parameters	Mean ± SEM		
		Control	Fluoride	Bifenthrin
1	Mean weight of eye (mg)***	233.31± 7.42^{a}	169.4± 1.05^{b}	118.26± 8.64^{c}
2	Mean volume of eye(μl)*	246.67± 5.2^{a}	264.5± 2.14^{b}	256.69± 17.78^{c}
3	Mean density of eye(mg/μl)***	0.9628± 0.05^{a}	0.64± 0.009^{b}	0.46± 0.002^{c}
4	Mean width of cornea (μ)***	169.31 + 13.8^{a}	128.65 + 7.46^{a}	272.52 + 23.63^{b}
5	Mean length of lens(μ)*	1234.51 + 32.76^{a}	1377.86 + 63.61^{ab}	1430.12 + 63.52^{b}
6	Mean width of lens(μ)**	925.65 + 44.55^{a}	801.86 + 44.89^{a}	1120.62 + 58.26^{b}
7	Cell density of lens/100 μ***	1413.56 + 50.73^{b}	954.50 + 88.87^{a}	777.56 + 39.79^{a}
8	Mean width of pigmented epithelium(retina) (μ)	24.37 + 2.89^{a}	20.35 + 1.76^{a}	18.89 + 0.55^{a}
9	Mean width of nuclear &plexiform layer(retina) (μ)	85.58 + 3.04^{a}	97.82 + 9.24^{a}	102.92 + 4.21^{a}
10	Mean width of bipolar layer(retina) (μ)*	28.84 + 1.00^{a}	24.11 + 2.59^{ab}	20.92 + 1.55^{b}
11	Mean width of ganglionic layer(retina) (μ)**	22.48 + 1.62^{a}	30.85 + 2.85^{b}	23.35 + 0.69^{a}
12	Mean width of nerve fiber layer(retina) (μ)*	12.47 + 0.56^{a}	10.99 + 1.29^{b}	12.43 + 0.79^{a}

‡: (Analyzed by one way ANOVA test), *: ($p \leq 0.05$), **:($p \leq 0.001$), *** :($p \leq 0.0001$).
Three groups not sharing a common lower case superscript differ significantly ($p \leq 0.05$) with each other.

project indicates a vital need of further in-depth studies of in-ovo insecticide exposures in general and OFI in particular to unearth organ and neurodevelopmental disruption in birds and mammals.

Ethics declarations

It is declared that all the research meets ethical guidelines and adheres to the legal requirements of Pakistan.

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CRediT authorship contribution statement

Sadia Suleman: Perform experimental work. **Fiza Azhar:** Perform experimental work. **Rabia Jabeen:** Perform experimental work. **Syeda Nadia Ahmad:** Help in manuscript write-up. **Khawaja Raees Ahmad:** Help in manuscript write-up. **Iram Inayat:** did proof reading & literature survey. **Zubedah Khanum:** did proof reading & literature survey. **Syeda Ayesha Ahmed:** did proof reading & literature survey. **Ayesha Faisal:** helped in Statistical analysis. **Sidra Yasmeen:** helped in Statistical analysis. **Muhammad Ali Kanwal:** helped in Statistical analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2023.09.014.

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