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Histo-anatomical mutilations of developing chick brain induced by in-ovo fluoride and bifenthrin exposure

Ayesha Faisal [a,1], Zubedah Khanum [a], Syeda Nadia Ahmad [b,*], Khawaja Raees Ahmad [a], Asma Younis [a], Sadia Suleman [a], Iram Inayat [a], Muhammad Ali Kanwal [a]

[a] Department of Zoology, University of Sargodha, Sargodha 40100, Pakistan
[b] Department of Zoology, University of Chakwal, Chakwal 48800, Pakistan

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ABSTRACT

Comparative developing brain histo-anatomical pathologies of Fluoride ions and Bifenthrin in-ovo exposures were explored in the golden black variety of domestic chick. Three exposure groups were –the Vehicle control group (Vg); Fluoride (F) group and the Bifenthrin (Bn) group each with forty fertilized eggs and received their respective group treatment at zero day of incubation. Embryos were extracted, dissected from head region and the embryonic whole brains were recovered after 14 days of incubation. The embryonic brains were preserved in bouin fixative for 24 h for further studies. The morphological results show the atrophied and hypertrophied embryonic brain in F and Bn groups respectively as compared to Vg group. The toxicological signs of encephalic anatomy and histology of F and Bn exposure were the enlarged third ventricles, optocoeles and arachnoid mater, encephalic spongiosis and decreased neuroglial density. The morphometric data showed significant decrease ($p \leq 0.05$) in mean weight and density of whole brain in F and Bn groups compared Vg. The mean length and width of whole brain in F were significantly lower than that of the Bn and Vg. whereas, the mean breadth of third ventricle in Bn remained significantly lower than F and Vg groups. On the other hand, the mean breadth of optocoele and fourth ventricle in F and Bn groups remained significantly higher than Vg. Conversely the mean optic lobe wall thickness in F remained significantly lower than Bn and Vg. Additionally, the mean neuronal density in diencephalon, optic lobe and cerebellum in F group and Bn group remained significantly ($p \leq 0.05$) lower than Vg. Results show that low dose in-ovo fluoride or bifenthrin exposure may cause neuro-developmental abnormalities in the developing chick embryos indicating that the Fluoride-ions and Bifenthrin harbor strong developmental neurotoxic capacity.

1. Introduction

Fluorine belongs to the halogen group that is commonly present in drinking water and toothpastes as fluoride ions [1]. Fluoride in drinking water is globally affecting over 200 million people and more than 25 nations in the world [2]. Fluoride is present naturally at different levels in rocks, water, and soil due to its high receptivity [3,4]. It is one of the elements essential for animal health; however, chronic exposure to large amounts of fluoride increases body burden in animals as well as humans [5]. Poultry animals are widely exposed to fluoride through water intake, soil particles and high fluoride commercial feed. High dose of fluoride induce kidney, liver, bone abnormalities, increase lipid peroxidation and disrupt anti oxidative system of broiler chickens. It also affects duodenal contractions and distort villus structure of duodenum in chicken [6]. Bifenthrin a fluoridated insecticide is strongly lipophilic and not only targets the nervous system of insects [7] but also disturb the development of non-target organisms [8], particularly domestic animals such as chick through oral exposure via contaminated feed [9]. Avian species are particularly threatened by the commonly used pesticides, leading to its decline and causes a potential hazard to human health due to its accumulation in meat and eggs [10].

Neuronal toxicity of fluoride and bifenthin is not observed in animals only but in humans as well, a recent study has shown lower intelligence quotient in children living in area with high fluoride in environment [11]. Fluoride intoxicated rats showed decreased neurotransmitter activity and increase in oxidative stress markers with reduced enzymatic

* Corresponding author.
E-mail address: nadia.ahmad@uoc.edu.pk (S.N. Ahmad).
[1] Authors contributed equally

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and non-enzymatic antioxidants which led to DNA damage and cell death in hippocampus of their brain [12,13]. Salmon exposed to relevant concentration of bifethrin causes alterations in metabolites muddled in proper neuronal function. The regeneration and branching of axons, quantity of corticospinal axon, and cell viability were among the top disease [14].

Fluoride and bifenthrin both are potential neurotoxic substances that can easily cross the blood brain barrier and accumulate in the brain tissue leading to multiple neuroglial damages by building up oxidative stress [15–17]. Oxidative stress is a result of distorted antioxidative/pro-oxidative balance and leads to biological retardations, and is considered as one of the main reasons of several pathological effects on poultry growth [17] Such damages mainly belong to the anatomical and physiological distortion of neurons [18,19]. Despite of antioxidant defense system these toxicological effects of fluoride and bifenthrin owe to an increase in the production of reactive oxygen species (ROS) [20,21]. The ROS, leads to induction of lipid peroxidation [22] causes mitochondrial DNA and membrane damages [23–26]. It is found that other environmental toxins such as copper or arsenic could induce oxidative stress, inflammation and autophagy in chicken brain [27]. Nerve damage, local hemorrhage, inflammatory infiltration and mitochondrial damage turned out to be flashing in histological results of copper and arsenic toxicity in chick brain [28].

Both fluoride and bifenthrin are reported developmental disruptors and harbor teratogenic potentials [29,30]. However little is known about the neuro-developmental toxicity of these teratogens especially from the point of view of the developmental anatomy of the brain. In present study the histo-anatomical disfigurements of developing chick brain upon in-ovo exposure of fluoride and bifenthrin are evaluated and compared.

2. Materials and methods

2.1. Egg collection and treatment

Fresh fertilized eggs (laid in preceding night) of golden-black variety of domestic chick (*Gallus domesticus*) were collected from small poultry breeders in the vicinity of Sargodha city. All the selected eggs (120) weighing **50 ± 2 g** were randomly distributed in three groups forty eggs in each group: (i) the vehicle control (Vg) group treated with **0.1 mL** of 5% dimethyl sulfoxide (DMSO-**Sigmaaldrich**) in demineralized water (DMW); DMW is used to nullify the effect of DMSO and same solvent of stock solution which is used for other groups as well (ii) the Fluoride group (F group) treated with 0.1 mL 5% DMSO + 0.01 mg/kg Fluoride solution **(i-dental)** in DMW (iii) the Bifenthrin group (Bn group) eggs were treated with 0.1 mL 5% DMSO + 0.01 mg/kg Bn in DMW.

2.2. Dose administration and incubation

Egg shells were cleaned carefully by rubbing with a cotton swab damped in 70% alcohol. A small area of egg shell was softened with a small droplet of concentrated HCl-**(Ittehad chemical limited)** applied with the help of a micropipette-**(Thermo Fisher)** in the middle of the two tapered ends. Each egg was placed horizontally (window facing laterally) for 10 min, so that the embryo (germinal disc) should gradually migrate on the top of the yolk ball and avoided from any injury. A fresh disposable insulin syringe (**1 mL**) with stainless steel needle of **12 mm** length was used to inject the relevant dose volume containing the required ingredients of the respective group through the soften egg shell area directly into the yolk ball in each egg. The dose was administered at zero day of incubation in all groups. Immediately after injection, the minute hole was properly sealed with melted wax to avoid contamination. Before incubation, weight of each egg was measured by electrical weight balance. The treated eggs of all three groups were placed in an incubator (Memmert) at **37 ± 0.5 °C** and 65% humidity for 14 days. During incubation all eggs were gently rolled manually, twice a day (12 hourly basis).

2.3. Embryo collection and micro-dissection for organ recovery

Eggs were candled daily to find out the embryonic development and growth. The eggs showing no perceivable embryonic development were treated as unfertilized and were replaced. The embryos were recovered by careful removal of the egg shells after 14 days. In this process the shell from the broader end of each egg was gently cracked to produce a wide hole. The inclusions were gently poured out into a china dish containing lukewarm normal saline water. The egg white (if any) was carefully removed away along with the extra embryonic membranes from the developing embryo. Intact brain from each embryo was finally dissected out by removing the slightly ossified skull with forceps and scissors. The embryonic brains were preserved in Bouin's fixative-**(Sigma Aldrich)** for 24 h and finally subjected to the morphological and morphometric studies prior to their serial sagittal and transverse-sectioning for further anatomical and histological investigations.

2.4. Morphological and morphometric observations

To show variations in the size and shape of the brain among the groups the digital snaps were captured in super-macro mode in Sony 7.2MP digital camera. The morphometric measurements such as weight, volume, density, and the length and breadth of the intact embryonic brains were obtained as follows. The organ weights were measured on a digital (0.001 mg precision) balance (Sartorius TE214S) while the volume were measured through liquid displacement method (**0.01 mL** precision) and finally the brain density (g/mL gram per milliliter) was calculated using the measurements of mass (weight) and volume in the given formula.

Density = Mass/Volume.

The length and width of whole brain was obtained with the help of a digital vernier caliper (with no zero error).

2.5. Histological preparations and studies

Whole brains were processed for gradual dehydration in 50, 70, 90% and absolute alcohol-**(Nedstar),** clearing in xylene-**(J.K. enteprises)** and wax **(Unilever Pakistan Limited)** embedding. Sagittal (the plane that divides the brain in right and left sides of brain) and transverse serial Section (3 micrometer thick) of selected embryonic brains of all three groups were obtained through rotary microtome **(Rotary Microtome, RMT-202; Bioevopeak).** These sections were carefully shifted on glass slides **(Thermo Fisher)** and then stained with Haematoxylin and Eosin **(Tissuepro)** for further histological and micrometric studies. The embryonic chick brain sections were meticulously observed to record all pathological anomalies. Digital photomicrographs were also captured in "Sony digital (DSC-W35)" **7.2 MP** digital camera adjusted on "Labomed CXR_2"trinocular research microscope. For micrometry these digital snaps were projected in corelDRAW11 under pre-calibrated scale.

2.6. Computer based micrometry

The micrometric data was generated, from digital photomicrographs (10x, 30x, 100x and 400x) of the 5 randomly selected histological sections of brain from each group in corelDRAW11. From each of 10x and 30x photographs the optic wall thickness, third ventricle and fourth ventricle width were calculated by using Bezier tool application on the snapshots. The optic walls cavity width was also calculated by using Bezier tool from the 100x photographs of sections of all groups. The neuron density of the Diencephalon, optic lobe and cerebellum was obtained from 4 different areas of each photograph with the help of (30 μm^2) quadrate at 400x. For calibration of the quadrate size and grid squares digital snapshot of the stage micrometer obtained on 10x, 30x, 100x and 400x respectively (keeping the camera specifications

unchanged) were used.

2.7. Statistical analysis

The morphometric (brain length, breadth, weight, volume, density and the breadths of 3rd and 4th verticals etc.) and micrometric (neuroglial cells density) data were analyzed statistically by using one way (ANOVA and Tukey's Multiple Range Test (TMRT). All statistical analyses were carried out employing IBM SPSS 20 statistics software.

3. Results

Various morpho-anatomical features and their departures were easily identifiable in the developing chick brains in Vg and the F and Bn groups respectively. In all three groups well developed cerebrum with properly placed inter-hemispheric fissure were observed. Behind the cerebral hemispheres a transverse fissure separated easily recognizable swellings called optic lobes. The pineal body was found located at a mid-posterior between the cerebral hemisphere while the cerebellum was located behind the pineal body and the optic lobes. A distinct medulla oblongata was situated behind to the cerebellum (Fig. 1).

The most common findings were the atrophied and hypertrophied embryonic brains in F and Bn groups respectively as compared to the Vg group. Analysis of the morphometric data shows significant ($p \leq 0.05$) decrease in mean weight and density of the entire embryonic brains in F and Bn groups; whereas the mean length, width and the volume of the developing chick brain Bn were significantly higher than that of Vg group (Table 1).

The sagittal histological sections taken from sagittal plane that divides the right and left side of brain, shows denser and more compact arrangement of neuroglial tissue in Vg group than that of F and Bn groups (Fig. 2, A1, B1 & C1). On the other hand, the transverse sections of the embryonic chick brains indicate enlarged third ventricle and medial encephalic region of the cerebrum in F and Bn groups as compared to the Vg group. Furthermore, the lateral ventricles were slightly enlarged and spongy in both F and Bn groups than that of the Vg group (Fig. 2, A2, and B2 & C2).

Significant ($p < 0.001$) decline in the mean breath of the 3rd ventricle with simultaneous increase in diameter of the 4th ventricle in both the F and Bn groups developing chick brains as compared to Vg were observed. Additionally, significant ($p < 0.05$) enlargement in the mean diameter of the optocoel (cavities of the optic lobes) was also observed in embryonic chick brains in both F and Bn as compared to the Vg group (Table 2).

The micrometry in terms of number of neurons per unit area in various regions shows most highly significant decline ($p < 0.0001$) in F and BF group in the Diencephalon, Optic Lobe (OL) and Cerebellum regions than that of the Vg (See Table 3).

Table 1
Morphometric parameters of the developing chick brains in Vehicle control, Fluoride and Bifenthrin groups.

Brain Parameters	Groups (Mean+SEM)	
	Vehicle control Fluoride	Bifenthrin
Length of chick brain (mm) * *	15.47 ± 0.708^{b} 9.264 $\pm 0.357^{a}$	16.14 ± 0.646^{b}
Width of chick brain (mm) * *	14.14 ± 1.005^{b} 8.23 $\pm 0.283^{a}$	15.14 ± 0.656^{b}
Weight of chick brain (g) *	0.2783 ± 0.010^{b} 0.2125 $\pm 0.054^{a}$	0.2459 $\pm 0.012^{ab}$
Volume of chick brain (mL) * *	0.2243 ± 0.021^{a} 0.3401 $\pm 0.011^{b}$	0.3029 $\pm 0.006^{b}$
Density of chick brain (g/mL) * *	1.3380 ± 0.143^{b} 0.6330 $\pm 0.061^{a}$	0.8171 $\pm 0.051^{a}$

SEM (Standard Error of the Means); *:$p \leq 0.05$, * *:$p \leq 0.0001$ (ANOVA); abc:Any two groups not sharing a common lower case superscript letter differ significantly ($p \leq 0.05$) with each other (TMRT)

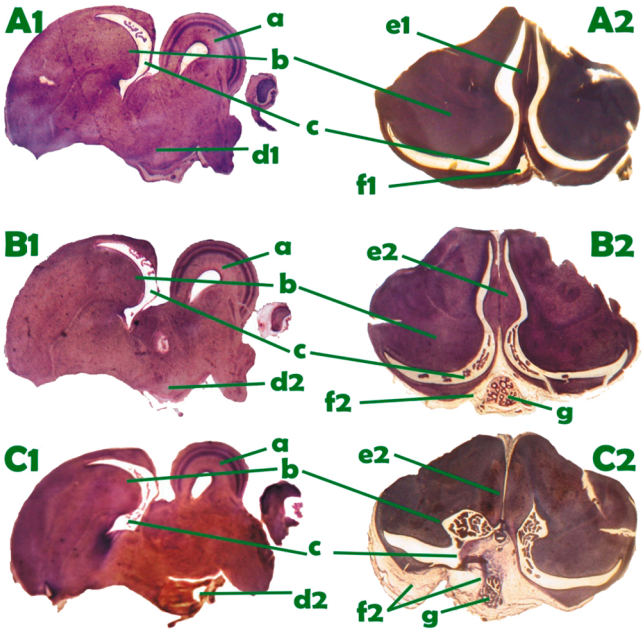

Fig. 2. Hematoxylin and eosin stained histological sagital (Left) and transverse (right) sections (10X) of chick brain. **A1,A2**: (Vg); **B1,B2**: (F); **C1,C2**: (Bn); a: Optic lobe, b: Cerebrum, c: Lateral ventricles, d1: Compacter region, d2: Lesions, e1: Normal encephalic region of cerebrum, e2: Swelled encephalic region of cerebrum, f1: Normal third ventricle, f2: Spongiosis, g: Myelin sheath formation.

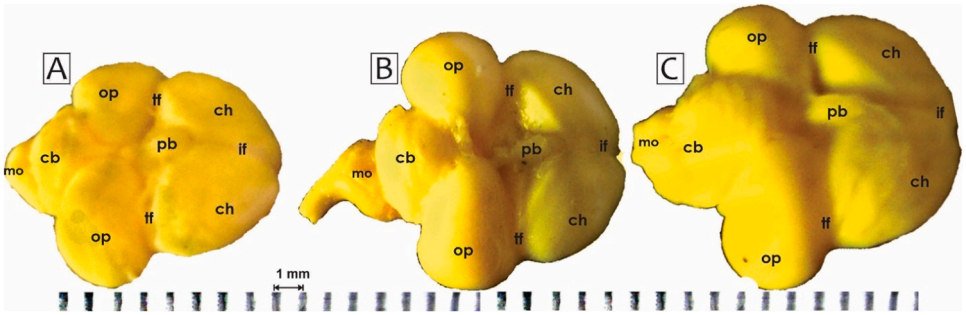

Fig. 1. : Dorsal view of chick brain. **A**: (F); **B**: (Vg); **C**: (Bn); ch: cerebral hemisphere, if: interhemispheric fissure, op: optic lobe,cb: cerebellum, tf: transverse fissure, mo: medulla oblongata, pb: pineal body.

Table 2
Histometric and micrometric parameters of ventricles of chick brain of Vg, F and Bn groups.

Parameters	Groups (Mean+SEM)	
	Vg F	Bn
Width of third ventricle (μm) * *	454.77 ± 9.61 ^{C} 355.12 ± 15.41^{b}	227.74 ± 26.65^{a}
Width of fourth ventricle (μm) * *	377.47 ± 11.96^{a} 390.42 ± 39.15^{a}	564.58 ± 13.41^{b}
Width of cavity of optic lobe (μm)*	371.004 ± 12.34^{a} 416.811 ± 9.23^{b}	417.804 + 10.35^{b}
Thickness of wall of optic lobe (μm)*	732.73 ± 20.00^{ab} 685.79 ± 10.46^{a}	760.50 ± 10.63^{b}

SEM (Standard Error of the Means); *:$p \leq 0.05$, * *:$p \leq 0.0001$ (ANOVA); abc:Any two groups not sharing a common lower case superscript letter differ significantly ($p \leq 0.05$) with each other (TMRT)

Table 3
Neuronal density per unit area (30 μm^2) of different parts of chick brain of Vg, F and Bn group.

Parameters	Groups (Mean ± SEM)	
	Vg F	Bn
Diencephalon * *	44.94 ± 0.2.00^{b} 27.26 ± 0.1.12^{a}	29.63 ± 1.21^{a}
Optic lobe * *	43.32 ± 1.43^{c} 19.79 ± 0.1.31^{a}	33.79 ± 1.92^{b}
Cerebellum * *	53.68 ± 1.74^{c} 34.47 ± 0.0.92^{a}	45.05 ± 1.02^{b}

SEM (Standard Error of the Means); * *:$p \leq 0.0001$ (ANOVA); abc:Any two groups not sharing a common lower case superscript letter differ significantly ($p \leq 0.05$) with each other (TMRT).

4. Discussion

Neuro-developmental toxicology is rather a new branch of toxicology where the brain and nervous system's development is studied under the influence of commonly found environmental toxins [31–33]. In this connection fluoride has been found to be a potent neurotoxicant that has caused behavioral, morphological and anatomical abnormalities [34]. The fluoride exposure has been found to cause neuronal fragmentations, vacuolizations, chromatolysis and retarded growth of cell bodies in mammals [35]. Like that of fluoride the organo-fluoridated fourth generation type 1 pyrethroid (bifenthrin) has also been reported highly neurotoxic in fish [36].

The present study was aimed to unearth the neuro-developmental toxicity of fluoride both as ionic and organic form at the morphological, histo-anatomical and the developmental histo-pathological level of the brain in developing chick. Unfortunately, there is an acute dearth of information in this area of research, thus the findings of this study cannot be directly compared with any existing information in the literature on the neurodevelopmental toxicity of avian system. So, it can be carefully claimed that this study may be the first ever report upon developmental neurotoxicity in avian system.

Various morphometric and anatomical observations of the developing chick brain indicate that fluoride ions can potentially retard embryonic brain development leading to regression in growth pattern as the significant decline in length, width and density is observed in treated groups embryo brains as compared to the control (untreated) group. Conversely the significantly increase in length, width and volume of the developing chick brains with a simultaneous decline in whole brain weight and density in the in-ovo Bn exposure group to that of the control embryonic brains indicate hypertrophy attributable to the embryonic neuro-encephalic edema. This data revealed that fluoride ions posing more toxicity than organo fluoridated bifenthrin. Enlarged encephaloceles resulting into bulged and swelled optic lobes and cerebral hemispheres enlarged lateral and 4th ventricles and dimensional changes in the 3rd ventricle indicate retention of the cerebrospinal fluid in the developing embryonic brains of both the F and Bn groups. The cerebral spongiosis both F and Bn embryonic brains may be a probably an indication of the developing brain neuroglial necrosis and depletion. Unfortunately, only a little is known on the micro-anatomical distribution and spatial organization of the different neuronal populations and their patterns of differentiation that form the encephalic vesicles and different synaptic layers of the developing chick brains. A slightly clear and enlightened picture of developing brain anatomy was put forward in 2016 [37].

The vestibular cavities or vacuolations seen in the medial encephalic region of the fore-brain may be attributable to the induced necrosis of the neuroglial stem cells [38]. In general, the lesser density observed in the vertical sections of the F and Bn treated chick embryo to that of the control group depicts the loss of neurons with reduced neuronal density in different regions of brain, in particular cerebrum and optic lobes also support this explanation and are thus the signs of neuroglial hypoplasia. As this was the pioneer study the interesting results obtained indicate the deeper explorations of the neurodevelopmental toxicity of fluoride exposure at molecular level to unearth the mechanistic processes involved and derailed by the fluoride exposure in such cases.

5. Conclusion

The results of the present comparative study have shown that fluoride and bifenthrin exposure both as ionic and organic forms at 0.01 mg/kg in-ovo on zero day in chick eggs may lead to specific histopathological and micro anatomical alterations. But in view of morphological results It can be concluded that fluoride ions in inorganic form were slightly more toxic than organo fluoride in bifenthrin molecule because bifenthrin contain few number of fluoride ions in it. Both are potentially developmental neurotoxic so their exposure through daily life sources should also be avoided to all possible extents during pregnancy in order to avoid neurodevelopmental anomalies.

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CRediT authorship contribution statement

Ayesha Faisal & Zubedah Khanum: Investigation, Data curation, Writing – original draft preparation, Formal analysis, Resources. **Syeda Nadia Ahmad:** Writing – review & editing, Visualization. **Khawaja Raees Ahmad:** Conceptualization, Methodology, Resources, Supervision, Validation. **Asma Younis and Sadia Suleman:** Formal analysis. **Irum Inayat:** Writing – review & editing. **Muhammad Ali Kanwal:** Writing – review & editing.

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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