COMPARATIVE TERATOLOGICAL OUTCOMES OF FLUORIDE IONS AND A FLUORIDATED INSECTICIDE (BIFENTHRIN) IN CHICK EMBRYOS

Zubedah Khanum,[a,†] Sadia Suleman,[a,†] Aqsa Mustanser,[a] Muhammad Waqar Ul Hassan,[a] Kausar Raees,[b] Muhammad Ali Kanwal,[a] Afia Zia[c], Khawaja Raees Ahmad[a,*]

Sargodha, Pakistan

.**ABSTRACT: Teratological capacities of fluoride ions (F), from NaF, and bifenthrin (BN) *in ovo* exposure were compared in the golden black variety of domestic chicken. Three groups (n=55) were studied: (i) vehicle control group (VC) with 0.1 mL 5% dimethyl sulfoxide (DMSO) in demineralized water; (ii) F group with 0.01 µg F/g egg weight (EW) and (iii) BN group with 0.01µg BN/g EW. The relevant doses were injected in freshly laid fertilized eggs. The embryos were removed from the egg shells after 14 days of incubation. The embryos were weighed and fixed in Bouin's solution for further studies. Morphological studies indicated general growth retardation in the embryos in the F and BN groups compared to the VC group. Reduced beak length, microphthalmia, exocardia, and meromelia were observed in the F group embryos whereas, different appendicular deformities such as fore-limb meromelia, contorted and polydactyl hind-limbs, un-clawed digits, and elongated digits corresponding to the index fingers were seen in the BN group embryos. Statistical analysis revealed a significant increase in dead and growth-retarded embryos in both the F and BN groups compared to the VC group. The morphometric data also showed a significant ($p \leq 0.05$) decrease in mean embryonic weight (F: 5.36±0.17 g, BN: 5.27±0.16 g; VC: 6.82±0.13 g), crown-rump length (F: 45.78±0.25 mm, BN: 44.30±1.15, VC: 51.67±0.81), and occipito-frontal length (FE: 13.92±0.18mm, BN: 12.65±0.14, VC: 16.05±0.13), in the FE and BN groups compared to the VC group. The findings indicate that fluoride is a potent disruptor of avian development both in ionic and organic forms.**

Keywords: Bifenthrin; Chick; Fluoride; Teratology.

INTRODUCTION

Poultry is a growing sector for the provision of meat and eggs to the whole world. Unfortunately, poultry have shown a susceptibility to the pesticides commonly used in poultry operations as well as to insecticide residues present in commercially available poultry feed.[1] In addition, pesticide residues may accumulate in the meat and eggs and can potentially threaten consumer health.[2-3] Another point of concern is that the pesticides and their metabolites accumulated *in ovo*, impose risks of developmental abnormalities and even embryonic death.[4-7] In a similar way, inorganic environmental toxicants such as heavy metals and, in particular, the element fluorine, have shown adverse effects on various organs, reproduction, and development.[8-10] The reported general permissible limit for fluoride ions (F) in drinking water for general human consumption is 1.5 ppm. Beyond this limit, F may cause dental fluorosis and/ or skeletal deformities.[11]

Like fluoride ions, fluoridated-organics (such as bifenthrin) have been found to induce various toxicological manifestations.[12-14] Both F and fluoridated-organics induce oxidative stress in those exposed, leading to damage in tissues and organ.[15-18] Developmental toxicological effects of F and BN have been reported in fish and

[a]Department of Zoology, University of Sargodha, Sargodha Pakistan; [b]Principal, Government College for Women Farooq Colony Sargodha; [c]Agricultural Chemistry Department, University of Agriculture, Peshawar, Pakistan; [†]Authors having an equal contribution. *For correspondence: Khawaja Raees Ahmad, Professor, Department of Zoology, University of Sargodha, Sargodha Pakistan; E-mail: raees.ahmad@uos.edu.pk; k.r.ahmad@gmail.com

rodents.[13,19-22] The reported embryonic toxicology of BN exposure in mice includes general growth retardation (GGR) as indicated by decreased crown-rump length, appendicular length, and in the brain and eye circumferences.[19] The toxic effects of fluoride ions on mouse embryos include, along with the various indicators of GGR, microcephaly, open eyes, hydrocephaly, twisted hind limbs, kinky tail, and hind limb meromelia.[8] However, no published information exists that highlights the developmental toxicity of fluoride ions or fluoridated-organics in bird embryos. The aim of the current study was to study the teratogenicity of F and BN exposure *in ovo* on developing chick embryos.

MATERIALS AND METHODS

Egg collection, treatment and incubation: Fertilized eggs (freshly laid in the preceding night) of the golden-black variety of domestic chicken were collected at approximately 8 am from poultry breeders in the nearby villages of Sargodha city. Eggs of approximately equal size (weighing 48–52g) were selected and distributed into three groups (n=55 per group): (i) the vehicle control group (VC group) was treated with 0.1 mL 5% dimethyl sulfoxide (DMSO) in demineralized water (DMW); (ii) the fluoride-treated group (F group) with 0.01 µg/g F in 0.1 mL 5% DMSO+DMW; and the Bifenthrin (BN group) treated with 0.01 µg/g BN in 0.1mL DMSO+DMW. The eggs of all three groups were placed in an incubator (Memmert) at 37±0.5ºC and 65% humidity for 14 days. The eggs were gently rolled manually, three times a day (8 hourly) every day.

Preparation of FE ions and BN solutions: The desired quantities of fluoride ions (0.01 µg F/g EW) from technical grade NaF and similarly of BN (0.01 µg BN/g EW) from technical grade BN were dissolved in 5% DMSO+DMW such that each 0.1 mL of the respective solution contained 0.5 µg of the active ingredient (F or BN).

Dose administration: The egg shells were sterilized using cotton swabs moistened in 70% alcohol to remove contaminants and residual traces of feces under the flow of dry air so that the alcohol would rapidly evaporate and not leach into the egg. A small 0.5 mm spherical window was created in each egg through partially dissolving the shell by the application of 0.2 µL of concentrated HCL (with the help of a micropipette) in the middle between the two ends. The eggs were placed horizontally (the "window" facing on the left lateral side) to for 10 minutes so that the presumptive embryonic discs could rise on the top of the yolk mass inside each egg. A sterilized 1 mL syringe was used to administer the group specific 0.1 mL dose solutions (as narrated above) through the "window" directly into the yolk in each egg. The tiny hole produced at the site of injection in the shell membrane in the middle of "the window" was closed using a drop of molten wax. The process was completed in a laminar flow cabinet.

Embryo collection: Embryos were collected on the 15thday of incubation. For this purpose, the rounded, shield-like portion of shell from the broader end of each egg was removed using fine scissors and forceps with the egg held in a vertical position and the narrower end pointed towards the ground. During this activity the developing embryos (being lighter than the yolk) slowly came to the top of the yolk mass and were visible through the opening produced. The egg contents were poured in a wide Petri dish to separate each embryo from the top of the yolk, using scissors, forceps,

and camel hair brushes. After the removal of the amniotic fluid and extraembryonic membranes using lukewarm normal saline and a paper towel, each excised embryo was weighed on a digital (0.001 mg precision) balance (Sartorius TE214S). These embryos were carefully observed in order to note any morphological defects. They were finally fixed in Bouin's solution for morphometric measurements and additional organo-metric, histological, and micrometric analyses.

Morphometry and data analysis: The morphometric measurements for the occipito-frontal length, the bi-parietal distance, and the crown-rump length were obtained for each embryo with the help of a digital vernier caliper. The morphometric data were subjected to appropriate statistical analyses (Chi-square, ANOVA and Tukey's post hoc analysis) employing Softonic SPSS20 software.

RESULTS

Chick eggs Fertility index: Analysis of the data for the numbers of unfertilized eggs and live, dead, and growth-retarded embryos using the Chi-square test revealed a significant difference ($p \leq 0.05$) between the BN and VC groups while a highly significant difference ($p \leq 0.001$) was noted between the VC and F groups (Table 1).

Table 1. Comparisons of the numbers of embryos recovered (n) among the groups (VC group=vehicle control group, F group=fluoride-treated group, BN=bifenthrin-treated group)

Parameter	Group		
	VC group (n)	F group (n)	BN group (n)
Total eggs	55	55	55
Live embryos	28	14	18
Dead embryos	2	6	4
No embryonic development (presumed unfertilized eggs)	19	20	21
Stunted growth embryos	6	15	12
χ^2		28.553†	11.782*

χ^2: Chi square goodness of fit test compared to the control group: *$p \leq 0.05$; †$p \leq 0.001$.

Embryonic Morphology: Compared to the VC embryos, the morphological abnormalities observed in the F group embryos showed general growth retardation, exocardia, evisceration, and decreased development of the down plumage as well as abnormal development of the eyes, limbs, and mandible. The embryos in the F group frequently showed micrognathia with rudimentary or no beak development. Similarly, limbic meromelia and microphthalmia were also frequently seen in the F group embryos (Figure 1).

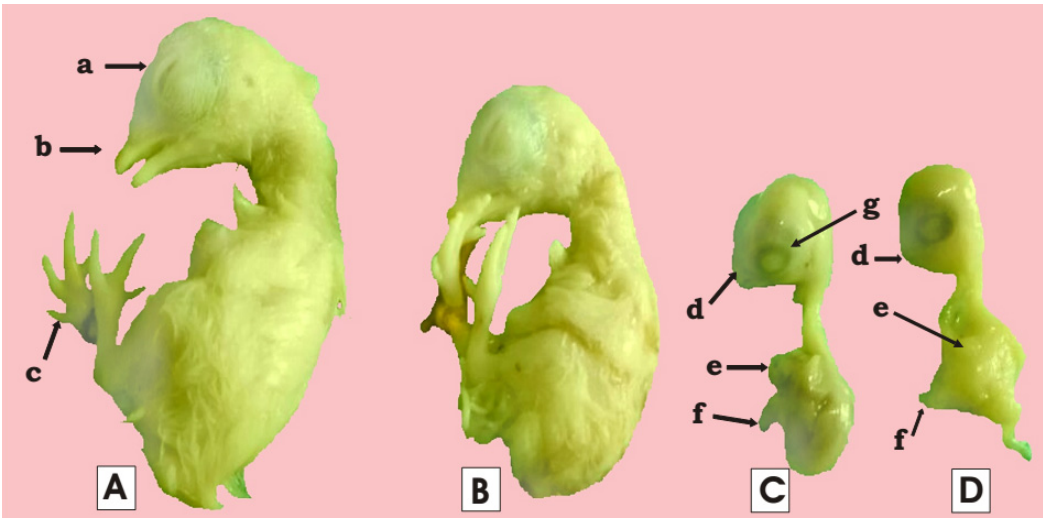

Figure 1. 14-day-old chick embryos showing different morphological anomalies involving reduced growth in the fluoride-treated group embryos (B, C, and D) as compared to the vehicle control group embryos (A). a: normal eye, b: normal beak, c: normal claws, d: reduced beak, e: exocardia, f: meromelia, and g: micropthalmia.

Apart from the general growth retardation that was observed, the morphological changes observed in the BN group embryos included appendicular deformities such as limbic meromelia, curved (bowed shaped) shanks, polydactyly (i.e., variations in the number of digits) in the hind limbs, prematurely opened eyes with congenital cataracts, and swollen peri-auditory meatus (opening of the external ears) (Figure 2).

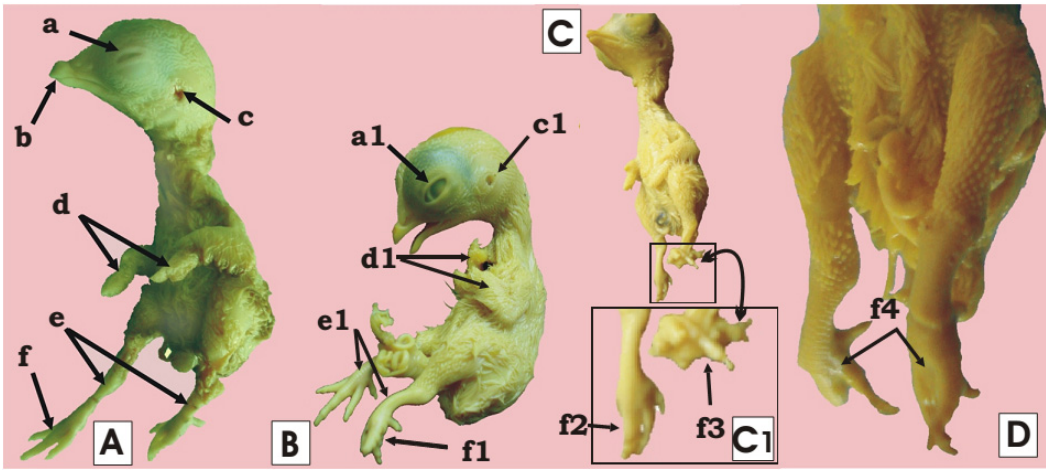

Figure 2. 14-day-old chick embryos showing different morphological anomalies in the bifenthrin-treated group embryos (B, C, C1, and D) as compared to the vehicle control group embryos (A). C1 shows a magnification of the hind-limbs of the bifenthrin-treated groups embryos. a: normal Embryonic Eye formation with eye lids, b: Normal Beak, c: External Auditory Meatus, d: Normal fore-limbs, e: normal hind-limbs, f: normal Hind-limb Digits, a1: open Eye showing cataract, c1: bulging external auditory meatus, d1: fore-limb meromelia, e1: smaller hind-limbs with bow-shaped shanks, f1: contorted hind-limb digits, f2: enlarged digit corresponding to the index finger and abnormally small digit corresponding to the little finger, f3: unilateral hind-limb polydactyly, and f4: contorted fingers.

Morphometric results: Statistical analyses (single factor ANOVA) revealed significant differences ($p \leq 0.001$) among the groups of mean values for the embryonic

weight, the crown-rump length, and the occipito-frontal length. These data were obtained only from live embryos of the three groups. Further post hoc analysis (Tukey's pair wise comparison) showed a significant difference ($p \leq 0.05$) between the VC and F groups, and also between the VC and BN groups, for the parameters of mean embryonic weight, the crown-rump length, and the occipito-frontal length but not for the bi-parietal length (Table 2).

Table 2. Variations in weight, crown rump-length, and morphometric readings of the skull of the developing chick embryo in the groups (VC group=vehicle control group, F group =fluoride-treated group, BN=bifenthrin-treated group; values are mean±SEM)

Parameter	Group		
	VC group (mean±SEM)	F group (mean±SEM)	BN group (mean±SEM)
Mean weight of embryo (g)*	6.82±0.13[a]	5.36±0.17[b]	5.27±0.16[b]
Mean crown-rump length (mm)*	51.67±0.81[a]	45.78±0.25[b]	44.30±1.15[b]
Mean occipito-frontal length (mm)*	16.05±0.13[a]	13.92±0.18[b]	12.65±0.14[c]
Mean bi-parietal length (mm)*	12.53±0.19[a]	11.7±00.19[a]	11.56±0.20[a]

*: For each parameter, the groups not sharing a common lower case superscript differ significantly from each other at the level of $p \leq 0.05$.

DISCUSSION

Bifenthrin belongs to the pyrethroid group of insecticides that are believed to be safe for vertebrates at low concentrations. The main feature of interest for this insecticide with respect to this study is the presence of fluoride in the insecticide. Inorganic fluoride alone has been found toxic to developing embryos as mentioned above. Systemic exposure in animal models and in humans can result in embryo lethality. In the case of human embryonic stem cells, F reduces viability and proliferation and induces cell death.[23] Thus it seemed imperative to study the toxicity to the embryo of fluoridated insecticides.

F exposure has been found to cause histopathology in various bodily organs including kidney, liver, and testes.[17,24] Similarly, BN, a fluoridated insecticide, has been found to be toxic to various bodily organs, including liver, kidneys, and lungs.[25, 26] Moreover, F and BN have also been found to possess embryo-toxic potential in zebrafish and rodent mammals. However, little is known about their teratological effects on birds. Development of the avian embryo is confined to the shell encapsulated environment and toxicants would need to gain entry into the egg on or before oogenesis to interfere in the developmental processes. Insectivorous and omnivorous birds may eat insects containing insecticides. In this way insecticides may accumulate in developing egg yolk during vitellogenesis. The insecticide bifenthrin contains organo-fluoride which is why we investigated its embryo-toxic potentials and compared them with those of fluoride ions in the avian system.

Our results indicated that F exposure during embryonic development caused higher embryonic mortality compared to BN while embyros exposed to BN exhibited higher

teratogenicity compared to those exposed to F. BN exposure has been found to induce various appendicular deformities along with swelling around the openings of the ears, and open eyes with indications of embryonic cataracts compared to the pathology observed in the F group embryo who had microphthalmia and micrognathia, scant down feather appearance, and rudimentary beak formation. However, both F and BN impeded the embryonic growth rate as indicated by the body mass index, the crown-rump length and the occipito-frontal length measurement parameters in 14-day-old chick embryos.

These findings indicate that water or food contaminated by the presence of the fluoride ion and/or BN, an organo-fluoride, may be deleterious for avian reproduction and embryonic development, especially if consumed at the egg laying stage as F or BN are deposited in the egg yolk during ovarian vitellogenesis. The most interesting finding in this study is that organo-fluoride (BN) exposure more severely interfered with the embryonic growth rate and also causes appendicular skeletal mutilations compared to inorganic fluoride exposure.

CONCLUSION

The findings of the present study indicate that *in ovo* fluoride (both inorganic or organic form) exposure (0.01 µg/g egg weight) can adversely affect appendicular, eye, and beak development along with causing general embryonic growth retardation in chicks. Fluoridated insecticides may be more potent developmental toxicants than fluoride ions alone. Thus, fluoridated insecticides should be used with extreme care.

ACKNOWLEDGEMENTS

The authors are thankful to the University of Sargodha for providing laboratory, animal house, financial, and administrative support for this research work. Thanks are also due to Dr Basharat Ali Saleem, Horticulture Officer, Department of Agriculture, Sargodha for providing 96% technical grade w/w BN (Batch No: Auc/ 20130611) with the courtesy of Auriga Chemical Enterprises, Lahore Pakistan.

REFERENCES

1 Holt PS, Davies RH, Dewulf J, Gast RK, Huwe JK, Jones DR, Waltman D, William KR. The impact of different housing systems on eggs safety and quality. Poult Sci 2011;90(1):251-62.

2 Jadhav VJ, Waskar VS. Public health implications of pesticide residues in meat. Vet World 2011;4(4):178-82.

3 Salar-Amoli J, Ali-Esfahani T. Determination of hazardous substances in food basket eggs in Tehran, Iran: A preliminary study. Vet Res Forum 2015;6(2):155-9.

4 Mufti SA, Nasim R. Avian embryo toxicity of dimicron. a commonly used insecticide. Biologia 1987;33:109-21.

5 Rao JV, Swamy AN, Yamin S, Rao SH, Rahman MF. Teratism induced in the developing chick by RPR–V, an organophosphate. Food Chem Toxicol 1992;30:945-51.

6 Lanselink DR, Midtling JE, Kolesari GL. Teratogenesis associated with oxydemeton methyl in the stage 12 chick embryo. Teratology 1993;48:207-11.

7 Wang CJ, Wang G, Wang XY, Liu M, Chuai M, Lee KKH. He XS, Lu DX, Yang X. Imidacloprid exposure suppresses neural crest cells generation during early chick embryo development. J Agric Food Chem 2016;64(23):4705-15.

8 Kanwal MA, Ahmad KR, Abbas T, Raees K, Suleman S Jabeen K. Alleviating properties of strawberry fruit pulp extract on fluoride-induced fetal anomalies in mice. Fluoride 2016;49(3 Pt 2):303-12.

9 Lin X, Gu Y, Zhou Q, Mao G, Zou B, Zhao J. Combined toxicity of heavy metal mixtures in liver cells. J Appl Toxicol 2016;36(9):1163-72.

10 Usuda K, Ueno T, Ito Y, Dote T, Yokoyama H, Kono K, Tamaki J. Risk assessment study of fluoride salts: probability-impact matrix of renal and hepatic toxicity markers. ″Biol Trace Elem Res 2016;173(1):154-160.

11 WHO. Fluoride in drinking-water: Background document for development of WHO guidelines for drinking-water quality. Geneva: WHO; 2004. Available from: http://www.who.int/water_sanitation_health/dwq/chemicals/fluoride.pdf

12 Jin M, Zhang X, Wang L, Huang C, Zhang Y, Zhao, M. Developmental toxicity of bifenthrin in embryo-larval stages of zebrafish. Aquat Toxicol 2009;95(4):347-54.

13 Liu J, Yang Y, Yang Y, Zhang Y, Liu W. Disrupting effects of bifenthrin on ovulatory gene expression and prostaglandin synthesis in rat ovarian granulosa cells. Toxicology 2011;282(1-2):47-55.

14 Al-Sarar AS, Abobakr Y, Bayoumi AE, Hussein HI, Al-Ghothemi M. Reproductive toxicity and histopathological changes induced by lambda-cyhalothrin in male mice. Environ Toxicol 2014;29(7):750-62.

15 Guo XY, Sun GF, Sun YC. Oxidative stress from fluoride-induced hepatotoxicity in rats. Fluoride 2003;36:25-9.

16 Chinoy NJ, Patel TN. The influence of fluoride and/or aluminum on free radical toxicity in the brain of female mice and beneficial effects of some antidotes [abstract]. Fluoride 2000;33(1):S8.

17 Sharma A, Chinoy NJ. Role of free radicals in fluoride-induced toxicity in liver and kidney of mice and its reversal [abstract]. Fluoride 1998;31: S26.

18 Jin Y, Pan X, Fu Z. Exposure to BF causes immunotoxicity and oxidative stress in male mice. Environ Toxicol 2014;29(9):991-9.

19 Zafar M, Asmatullah. Determination of LD50 and morphometric analysis induced by bifenthrin in developing fetuses of mice. Biologia (Pakistan) 2013;59(2):299-305.

20 Zafar M Asmatullah. Some studies on histopathological and cranio-facial structures in mice on co-gestational exposure of bifenthrin. Biologia (Pakistan) 2014;60(1):153-8.

21 Ahmad KR, Kanwal MA, Raees K, Abbas T. Strawberry Fruit extract ameliorates pregnancy and feto-gestational effects of sodium fluoride exposure in mice. Fluoride 2015;48(1):62-8.

22 Collins TFX, Sprando RL, Shackelford ME, Black TN, Ames MJ, Welsh JJ, Balmer MF, Olejnik N, Ruggles DI. Developmental toxicity of sodium fluoride in rats. Food Chem Toxicol 1995;33:951-60.

23 Fu X, Xie FN, Dong P, Li QC, Yu GY, and Xiao R. High-dose fluoride impairs the properties of human embryonic stem cells via JNK signaling. PLoS One 2016;11(2):e0148819.

24 Ahmad KR, Nauroze T, Raees K, Abbas T, Kanwal MA, Noor S, Jabeen S. Protective role of jambul (*Syzygium cumini*) fruit-pulp extract against fluoride-induced toxicity in mice testis: a histopathological study. Fluoride 2012;45(3):281-9.

25 Zhang Y, Lu M, Zhou P, Wang C, Zhang Q, Zhao M. Multilevel evaluations of potential liver injury of bifenthrin. Pestic Biochem Physiol 2015;122:29-37.

26 Dar MA, Raina R, Mir AH, Sultana M, Pankaj NK. Effect of dermal application of bifenthrin on acetylcholinesterase and oxidative stress indices in rat Blood, Lung and Kidney. Proc Natl Acad Sci India Sect B Biol Sci 2015;85(2):431-5.