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Ameliorative Potentials of Methylcobalamin (Vitamin B12) Against Teratogenic Effects Induced by Oxyfluorfen in Chick Embryo

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ABSTRACT

Purpose: Teratological potential of oxyfluorfen and the antioxidant role of Methylcobalamin was studied in the golden black variety of domestic chicken *Gallus domesticus*.

Methods: The study was conducted on 200 fresh fertilized eggs collected and divided into 4 groups as follows: (1) Control injected with 0.1 µL of 5% DMSO in corn oil (2) Oxy, injected with 0.1µL of 0.01µg/g oxyfluorfen solution in 5% DMSO and corn oil (3) MeCbl, injected with 0.1µL of 0.01µg/g methylcobalamin solution (4) OxyMeCbl, injected with 0.1µL of 0.01µg/g oxyfluorfen solution in 5% DMSO and corn oil and 0.1µL of 0.01µg/g methylcobalamin solution. In-ovo treatment was given on zero day and embryos were incubated & on 14th day of incubation, the embryos were recovered from eggs and fixed in fixative (90% alcohol and 10% formaldehyde) for further studies.

Results: The morphological observations showed a significant increase in mortality rate among the oxyfluorfen treated embryos against control group. Morphological analysis revealed various adverse effects, including reduced weight, crown-rump size, axial and appendicular skeleton size. Moreover, deformities were noted in the beak, eye, and neck formation. Cataracts were frequently observed in the eyes, and some embryos showed reduced head size with a prominent decrease in the beak size. Development of feathers was also affected, and several cases exhibited umbilical cord hernias. However, when the embryos were treated with methylcobalamin after oxyfluorfen exposure, there was a noticeable improvement in developmental effects.

Conclusions: Oxyfluorfen, containing fluorine causes birth defects in chick embryo that might be due to oxidative stress. Methylcobalamin has showed potential to act as antioxidant by countering oxyfluorfen's harmful effects

Key-words: *Oxyfluorfen; Methylcobalamin; Gallus domesticus; Morphological Abnormalities*

INTRODUCTION

Oxyfluorfen is a broad-spectrum diphenyl-ether herbicide from organochlorine pesticides class and is used for the control of broad leaf grassy weeds. Due to its hydrophobic nature it can accumulate in the fatty tissues and exhibits acute dermal, oral and inhalation toxicity in living organisms[1, 2]. The primary target sites where oxyfluorfen shows effects are liver and blood. It inhibits protoporphyrinogen oxidase enzyme to interfere with heme biosynthesis that causes anemia and hepatotoxicity[3]. It produces reactive oxygen species (ROS) that link with unsaturated fatty acids to damage cell membrane, disrupt endocrine genotoxicity and suppress sex hormones due to oxidative stress[4, 5]. They also cause several abnormalities in acanthoses, echinocytes and micronuclei to disrupt morphology of red blood cells[6]. Oxyflourfen contains fluoride that has potential to effect various body organs and levels of reproductive hormones that cause issues with fertility and development[7].

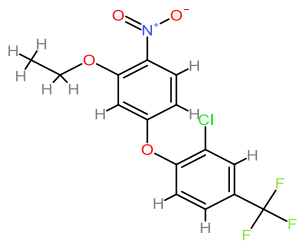

Figure 1 Chemical structure of Oxyflourfen

Vitamin B12 shows antioxidant properties by reducing superoxide levels in cytosol and mitochondria[8]. In female Long-Evan rates the levels of superoxide in ganglion cells of retina were lowered by administration of Vitamin B12[9]. Similarly in rats with B12 deficiency reduced epidermal growth factor levels and amplified tumor necrosis factor alpha were noted. It suggests that presence of vitamin B12 regulates the expression of cytokines and growth factors and reduces the inflammatory oxidative stress[10]. Many internal and environmental stresses are associated with production of ROS by disrupting redox reactions[11]. Tissues in chick embryo contain highly polyunsaturated fatty acids and need antioxidant defense[12]. The ROS directly influence the embryonic development by influencing the cell signaling pathways for proliferation, differentiation and apoptosis[13]. Moreover, B12 can scavenge the ROS directly by acting as antioxidant and modulating cytokines and growth factors. Also, it offers protection against oxidative stress induced by immune response. Therefore, it can have a direct effect on regulating the embryonic development by suppressing the ROS[14]. It has also been reported that in-ovo injection of B12 in fertilized eggs improve hatchability, feathering and growth rate and lowers the mortality rate.[15]

The current research aims to assess the potential teratogenic effects of oxyfluorfen on chick embryos and the potential of methylcobalamin in alleviating these effects. Chick embryos are an ideal experimental model for teratogenic investigations due to their ready availability, cost and time efficiency, and the ability to generate statistically significant results through scalable experimentation.

MATERIAL AND METHODS

Egg collection and dose preparation

For experimentation purpose, 200 freshly laid fertilized eggs of golden black breed of hen were collected from different villages near Sargodha, Punjab. Eggs were brought in Reproductive Endocrinology and Histopathology lab, University of Sargodha within 24h of collection. Eggs were weighed and those with approximately equal weight 42-45grams were selected and distributed into 1 control and 3 experimental groups randomly (50 eggs each).

a. **Control:** 0.1µL of 5% DMSO in corn oil was injected in e ach egg of control group.

b. **Oxy:** 0.1µL of 0.01µg/g oxyfluorfen solution in 5% DMSO and corn oil was injected in each egg of toxin group.
c. ***MeCbl:*** 0.1µL of 0.01µg/g methylcobalamin solution in water was injected in each egg. Solution was made in water because B12 is water soluble.
d. ***OxyMeCbl*:** 0.1µL of 0.01µg/g oxyfluorfen solution in 5% DMSO and corn oil and 0.1µl of 0.01µg/g methylcobalamin solution was injected in each egg.

Dose Administration

Eggs were cleaned and sterilized properly using a cotton swab dipped in diluted alcohol. Approximately 1 drop of concentrated HCL was dropped on the egg using a 1mL syringe to soften the egg shell and create a window for dose injection. The eggs were placed in horizontal position for 5-6 minutes to let the yolk rise at the top. With the help of sterilized micropipette tips dose was injected into the yolk sac of the fertilized eggs through the window to minimize contamination chances. After dose injection, the small window on the egg surface was sealed properly using melted wax to avoid any kind of contamination from environment. Each egg was weighed using electric balance after the wax got dried up and windows were sealed properly.

Eggs incubation

After dose administration, the eggs were kept inside the sterilized automatic egg incubator (Nanchang Vena egg incubator, VA-48) with capacity of 48 eggs. The temperature for incubation was set at 37°C and humidity at 60%. To keep the desired humidity level constant, water was refilled inside the incubator every 48 hours in winters and 24 hours during summers. The rotation of eggs was set to be done every 5 hours. Eggs were incubated for 14 days. Candling was done at regular intervals and eggs with no embryo development were discarded.

Embryo recovery

On 14th day, eggs were removed from the incubator and weighed using electric balance. Using forceps and scissors the broader end on the side of each egg was removed. Yolk being heavier than embryo went to bottom and the lighter embryo is exposed at the top. The embryos were carefully removed from the shell and were shifted to saline solution. Yolk was separated from embryo using camel hair brush and scissors. The extracted embryos were placed inside the fixative solution (90% alcohol and 10% formaldehyde) for 48h.

Morphometry

The embryos were removed from the fixative and weighed one by one on an electrical balance. Embryos were kept inside the petri dish with fixative to prevent drying out. Morphological deformities were noted by thoroughly observing the embryos. Morphometric measurements like crown-ramp length, beak length, eyes length and width and forelimbs length were taken using Vernier caliper with no zero error and saved for statistical analysis.

Statistical analysis

Data collected from the chick embryo samples was analyzed by applying ANOVA and ANCOVA test. IBM SPSS Statistics 23 software was used to run the tests

RESULTS

Morphological Results

Control group showed normal development in chick embryo. All morphological features including development of head, eyes, beak, fore limbs and hind limbs, upper body growth and lower body growth was normal (Fig. 1A). Oxy group in contrast to control group revealed abnormal effects of toxicity on chick embryos. Weight reduction and decrease in embryo size was evident. Microcephaly and anophthalmia were most common abnormalities. Embryonic cataract, abnormal beak development, skewed spine and why neck was noted due to which head was tilted from the normal angle. Appendicular abnormalities including phocomelia, meromelia, torsion in shank and atypical development of digits was noted. Mortality rate was higher in Oxy group as compared to control group (Fig. 1B). Chick embryos of MeCbl group showed increase in growth, weight and overall development (Fig.1C). Meanwhile, OxyMeCbl group showed minor reduction in size than control group. Other anomalies occurrence was also suppressed by presence of vitamin B12. Morphological features almost resembled the control group which means B12 have promising effects against pesticide exposure (Fig. 1D).

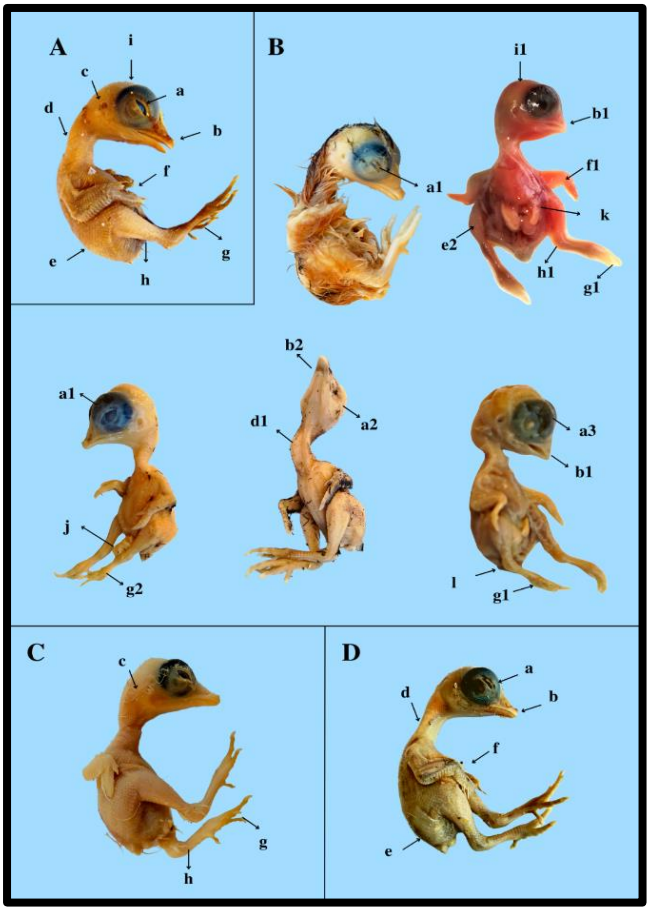

Figure 1: (A) Control, (B) Oxy, (C) MeCbl, (D) OxyMecbl, a: normal eye formation, a1: widely opened eye and embryonic cataract, a2: anophthal mia, b: Normal beak, b: normal beak, b1: reduced beak or agnathia, b2: enlarged lower beak, c: Normal Auditory Meatus, d: normal neck, d1: wry neck, e: normal down development, e2: reduced down development, f: normal forelimbs, f1: meromelia, g: normal hind limbs, g1: hind limbs meromelia, g2: phocomelia h: normal shank, h1: reduced shank, i: normal head, i1: microcephaly, j: umbilical cord hernia, k: hemorrhagic body, l: muscular dystrophy.

Morphometric Results

Mean weight of embryo showed a highly significant increase (P≤0.001) in OxyMeCbl group as compared to Oxy group. Crown ramp length in MeCbl and OxyMeCbl group was significantly higher against control group. Similarly the front occipital length, biparital length, eye width and length, beak length, brachium and anti-brachium length was significantly decreased in Oxy group as compared to control group. However, these parameters were significantly (P≤ 0.001) increased in OxyMeCbl group as compared to Oxy treatment group. Moreover, shank length, thumb length, index finger, middle finger and little finger length showed a significant (P≤ 0.05) decrease in Oxy group and increase in OxyMeCbl group as compared to control group. However, thumb length showed no significant difference in any group (Table 1).

Table 1. Variation between measurements of morphometric parameters on 14th day in chick embryo (**Oxy:** Oxyfluorfen, **MeCbl:** Methylcobalamin**, OxyMeCbl:** Oxyfluorfen+Methylcobalamin)

Morphometric parameters	Mean + SEM			
	Control	Oxy	MeCbl	OXYMeCbl
Mean embryo weight (g)***§	8.64±0.35[a]	4.73±0.53[b]	8.79±0.39[a]	8.25±0.36[a]
Mean crown-ramp length (mm) ***§§	49.11±0.95[a]	37.29±3.28[b]	51.38±1.33[a]	49.26±1.22[a]
Mean fronto-occipital length (mm)*§	16.97±0.81[a]	13.32±1.22[a]	19.29±0.91[a]	17.41±0.81[a]
Mean biparital distance (mm)§	12.49±0.37[ab]	11.09±0.56[b]	12.72±0.41[a]	13.14±0.37[a]
Mean eye width (mm)*§	10.16±0.85[a]	4.12±1.29[b]	11.18±0.95[a]	11.91±0.87[a]
Mean eye length (mm)*§	10.53±0.90[a]	4.17±1.37[b]	11.74±1.01[a]	11.59±0.91[a]
Mean eye circumference (mm)*§	8.68±0.52[ac]	5.42±0.77[bc]	9.94±0.57[ab]	7.52±0.51[a]
Mean upper beak length (upper) (mm)**§	10.46±0.52[a]	6.01±0.79[c]	13.17±0.58[b]	11.05±0.52[a]
Mean lower beak length (mm)**§	10.06±0.44[a]	5.784±0.67[b]	10.94±0.49[a]	9.985±0.45[a]
Mean anti-brachium length (left) (mm)**§	10.1±0.32[a]	5.50±0.47[b]	10.93±0.35[a]	10.36±0.32[a]
Mean anti-brachium length (right)(mm)***§	10.15±0.47[a]	5.40±0.71[b]	11.12±0.53[a]	10.25±0.47[a]
Mean brachium length (left) (mm)*§	9.20±0.40[a]	6.23±0.61[b]	10.35±0.49[a]	9.89±0.40[a]
Mean brachium length (right) (mm)***§	9.50±0.34[a]	6.09±0.51[b]	10.77±0.38[ac]	10.04±0.34[a]
Mean shank length (left) (mm)*§	10.35±0.40[b]	8.83±0.60[b]	11.83±0.45[a]	11.65±0.40[a]
Mean shank length (right) (mm)*§	10.69±0.37[a]	8.67±0.56[b]	11.63±0.41[a]	11.17±0.37[a]
Mean thumb length (left) (mm) §	4.07±0.35[ab]	3.26±0.55[ab]	5.22±0.41[a]	4.71±0.37[a]
Mean thumb length (right) (mm) §	4.22±0.33[ab]	3.30±0.50[b]	5.21±0.37[a]	4.58±0.33[a]
Mean index finger length (left) (mm)**§	8.76±0.47[a]	6.26±0.72[c]	11.56±0.53[b]	9.69±0.48[a]
Mean index finger length (right) (mm)*§	8.81±0.57[a]	6.07±0.87[c]	11.41±0.64[b]	9.28±0.58[a]
Mean middle finger length (left) (mm)* §	8.77±0.47[c]	6.26±0.72[a]	11.56±0.53[b]	9.69±0.48[a]
Mean middle finger length (right) (mm) *§	8.81±0.57[c]	6.07±0.87[a]	11.40±0.64[b]	9.28±0.57[a]
Mean little finger length (left) (mm)*§	8.52±0.54[a]	5.77±0.81[a]	10.10±0.59[b]	7.46±0.54[a]
Mean little finger length (right) (mm) *§	8.69±0.51[a]	5.43±0.77[a]	9.94±0.57[b]	7.52±0.51[a]

*: P≤ 0.05, **: P≤ 0.001, ***: P≤ 0.0001, §: Analyzed by ANCOVA, §§: Analyzed by ANOVA

DISCUSSION

Teratogenic potenials of oxyflourfen have been reported in several studies. In Egyptian Nile fish, oxyfluorfen induces HSP 70 protein in kidney and liver and can inhibit the acetylcholinesterase activity in the brain of *Gambusia affinis* and *Orechromis niloticus*[16, 17]. Study of oxyfluorfen toxicity in Zebra fish indicated liver damage by obstructed hepatocyte proliferation and inflammation due to interference with glycolytic function and lipid metabolism. Yolk edema and shortness of body length was also noted[18]. Investigation of effects on Japanese Medaka fish has revealed that oxyfluorfen effects on growth and development of the embryonic stages at different concentrations. Moreover, it effects larval growth and morphology by interfering with gene expression regulation at transcription level[19]. Another study revealed that oxyfluorfen induces liver damage, kidney impairment, hematological alterations, endocrine disruption genotoxicity, disruption of sex hormones and oxidative stress in African cat fish at low concentrations[5, 20].

In present study, oxyfluorfen at very low dose 0.01µg/g caused mortalities, skeletal deformities of axial and appendicular skeleton and umbilical hernia in chick embryos. Limbs development, eyes development, retardation in growth and disruption in normal development of lower skeletal elements like claws was highly effected which means that the embryonic tissues synthesis and bone formation was highly affected by exposure to oxyfluorfen. Fluorine containing pesticides interfere with tissues and organs by generating ROS and cause oxidative stress that leads to skeletal deformities[21]. Results from the present study can suggest that oxyfluorfen containing fluorine has ability to cause oxidative stress that can interfere with normal embryonic development in chick embryo.

Previous studies in hens have revealed the increase in body growth and weight in chicks with B12 injection[22]. Study of in-ovo feeding of Vitamin B12 in broiler chicken revealed increased levels of proteins and glucose[23]. The levels of albumin increased too. Vitamin B12 also had positive effects on hatchability. B12 supplementation to hens decrease the fat content in liver and reduces the affinity for ROS[24]. In a study based on Rhode Island Red Hens injection of Vitamin B12 in deficient hens improved hatchability. Chicks injected with B12 showed higher growth rate and feathering. The mortality rate was significantly decreased[15].

The present study reveals the antioxidant properties of vitamin B12 methylcobalamin against teratogenic effects induced by oxyfluorfen. Oxyflourfen is highly involved in production of ROS that may have caused teratological effects in chick embryos. The positive effects of this water soluble vitamin provides shield against the reactive oxygen species by inhibiting ROS production that effect normal functioning of body tissues and reproductive development [25].

Chicks that feed on worms or plants contaminated with oxyfluorfen can be saved from lethal effects by B12 dosage. Moreover, it can be predicted that adding vitamin B12 in diet can reduce the risks of abnormalities induction in humans who are exposed to harmful agents like pesticides[26].

CONCLUSIONS

Oxyfluorfen treatment can harm the growth of bird embryos, causing birth defects due to oxidative stress. But we need more detailed research to understand how exactly this damage happens and the role of ROS in messing up development. However, a study suggests that giving methylcobalamin to chick embryos exposed to oxyfluorfen can reduce these birth defects.

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Not Applicable.

CONFLICT OF INTERESTS

None

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