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Protective role of Pyridoxine (Vitamin B6) against teratogenic effects induced by Oxyfluorfen in chick embryo

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ABSTRACT

Purpose: This study evaluated the embryotoxic and teratogenic effects of oxyfluorfen (OXY) and the protective potential of pyridoxine (vitamin B6) in chick (Gallus domesticus) embryos.

Methods: Two hundred fertilized eggs were randomly distributed into four groups (n=50/group) and injected with 0.1 µL of solution in 5% dimethyl sulfoxide (DMSO) on incubation day 0: Control (5% DMSO vehicle), Oxyfluorfen (OXY; 0.01 µg/g), Pyridoxine (B6; 0.01 µg/g), & Oxyfluorfen + Pyridoxine (OXY+B6; 0.01 µg/g each). Embryonic development was assessed morphologically on day 14.

Results: Control embryos exhibited normal size, weight, crown-rump length, and morphology. OXY exposure caused significant ($p < 0.05$) embryotoxicity, including increased mortality, reduced weight (5.55±0.86 g), and severe teratogenic effects: anophthalmia, cataracts, cervical scoliosis, subcutaneous hemorrhaging, muscular dystrophy, limb malformations (amelia/phocomelia), and incomplete ventral body wall closure. Co-administration of pyridoxine significantly attenuated OXY toxicity; the OXY+B6 group showed improved embryonic weight (6.53±0.77 g) and crown-rump length (43.42±4.03), along with reduced severity of muscular dystrophy and limb bone thinning compared to the OXY group.

Conclusions: These results demonstrate that OXY disrupts normal chick embryonic development, while pyridoxine provides substantial protection against its embryotoxic and teratogenic effects.

Keywords: *Chick embryo, Oxyflourfen, Vitamin B, Teratogens*

INTRODUCTION

Oxyfluorfen (OXY) is an organo-chlorine herbicide [1] which is activated by light. [2] It increases the level of reactive oxygen species within a cell [3] which results in lipid peroxidation of cell membranes [4]. Due to its high affinity for lipids and hydrophobic characteristics, it can accumulate in fatty tissues of animals [5]. Its chronic exposure in rodents results in variegate porphyria, hyperplastic nodule formation, and necrosis [6], while acute poisoning leads to a decreased respiratory rate and concurrent skin irritation [7]. In human oxyflourfen is reported to cause blood related toxicity. It causes moderate to low cytotoxicity in rat thyroid follicular cells [8].

OXY has been linked to scoliosis-like morphometric deformities and a reduction in body length [9], attributed to its inhibitory effects on skeletal growth of fish [10]. Moreover, its exposure during pregnancy may result in congenital anomalies in fetus such as transverse limb deficits or craniosynostosis in human [11]. Its toxicity is also associated with the deterioration of connective tissue and may result in chemical burns affecting the forearm fascia in human [12].

Vitamin B6 plays an important function as coenzyme in a multitude of intricate cellular processes [13]. It has assumed a critical role in the meticulous regulation of cellular redox equilibrium [14] and manifests neuroprotective attributes [15]. It stands as a therapeutic remedy for addressing pyridoxine-dependent seizures

and acts as a protective agent against congenital cardiovascular malformations in the developing fetus. Vitamin B6 exerts a notable influence on the augmentation of red blood cell count and the elevation of hemoglobin concentration [16]. It concurrently enhances physiological parameters such as body weight and protein retention. Furthermore, empirical evidence derived from investigations involving in ovo vitamin B6 administration reveals a notable enhancement in chick embryo development, as exemplified by research conducted on turkey poults [17].

In considering the toxic aspects of OXY and protective potentials of Vitamin B6, the present study was conducted to check the teratogenic effects of OXY for Gallus domesticus embryos and protective potentials of Vitamin B6 against these damages.

MATERIAL AND METHODS

Test substances, Experimental groups and dose preparation

Fertilized eggs of the golden-brown chick breed (Gallus domesticus) were collected within a 24-hour period from rural areas in Sargodha. In total, 200 eggs were used, each weighing approximately 38-50 grams. Based on prior research on chick embryos and nutritional requirements, the in ovo dose of OXY (0.01 µg/g) and B_6 was chosen to ensure physiologically realistic exposure while avoiding non-specific developmental damage.

Eggs were equally distributed in four groups (50 each) according to treatment injected at zero day of study. i) Control group (CG): injected with 0.1 ml of 5% DMSO solution in water; ii) OXY group (OxyG): Injected with 0.1ul of 0.01ug/g OXY solution in 5% DMSO; iii) Vitamin B6 group (B6G): Injected with 0.1ul of 0.01ug/g vitamin B6 solution in 5% DMSO; iv) OXY+Vitamin B6 group (OxyB6): Injected with 0.1ul of 0.01ug/g OXY+ 0.01ug/g vitamin B6 solution in 5% DMSO.

Dose administration

The eggs were sterilized and cleaned with 70% alcohol using cotton swabs followed by air drying. A small drop of concentrated HCl solution was applied to create a window in the broader end of the eggshells. Eggs were maintained in a horizontal position for 4-5 minutes to allow the embryo to rise to the top. Group specific treatment was administered through the windows into the yolk sac of each egg using a sterilized syringe. After that melted wax was applied to seal the windows. Prior to incubation, the weight of each egg was recorded using an electronic balance.

Incubation

After dose administration, the eggs were incubated in a sterilized Nanchang Vena Egg Incubator VA-48, which has a capacity of 48 eggs. Standard conditions of temperature (37.5 ℃) and humidity (60%) were maintained. Eggs were rotated after every 6 hours, and a daily candling process was executed to identify and eliminate unfertilized and deteriorated eggs.

Recoveries and morphometry of embryos

On the 14th day of incubation, embryo recoveries were carried out. Each egg's weight was measured, and the broader side of the eggs was removed with forceps and egg contents were transferred in saline solution. Camel hairbrushes and forceps were used to isolate embryos from the yolk sac. Weight of each embryo was measured using an electronic balance. A mixture of 10 ml of formaldehyde and 90 ml of alcohol was used as a fixative, and embryos were fixed for 48 hours.

Vernier caliper was used for measurement of morphological parameters including crown-rump length, fronto-occipital length, eye dimensions, bi-parietal distance, and limb lengths for all four groups. Selected embryos from each group were photographed using a 16MP camera in super macro mode to study and compare morphometric and skeletal developments of embryos in different groups.

Statistical analysis

The morphometric measurements were statistically analyzed by one way ANOVA and ANCOVA test in IBM SPSS Statistics 22 software. Data for crown-ramp length was analyzed through one way ANOVA, while data for all other parameters were analyzed by ANCOVA and crown-ramp length was taken as covariat. Crown ramp is the length of full embryo. Therefore, it is used as covariate for other parameters.

RESULTS

Morphological Results

In the control and B6G groups embryos exhibited normal body development marked by standard size and weight parameters. The morphological features including crown-rump length, eye, beak, fronto-occipital dimensions relevant to bi-parietal head size, upper and lower limb as well as the unperturbed progression of down feathers were normally developed. In OxyG group, a notable reduction in both body weight and embryo size was observed. Among morphological parameters limb and beak development were particularly adversely affected. Furthermore, a range of abnormalities were noted, including anophthalmia (absence of eyes), protruding eyes, neck misalignment, the presence of hemorrhagic spots on the skin coupled with muscular dystrophy. Appendicular deformities were identified, such as amelia or phocomelia (absence or underdevelopment of limbs), limb torsion, abnormal phalangeal growth or complete phalangeal absence, and incomplete closure of the ventral body wall. These findings underscore the

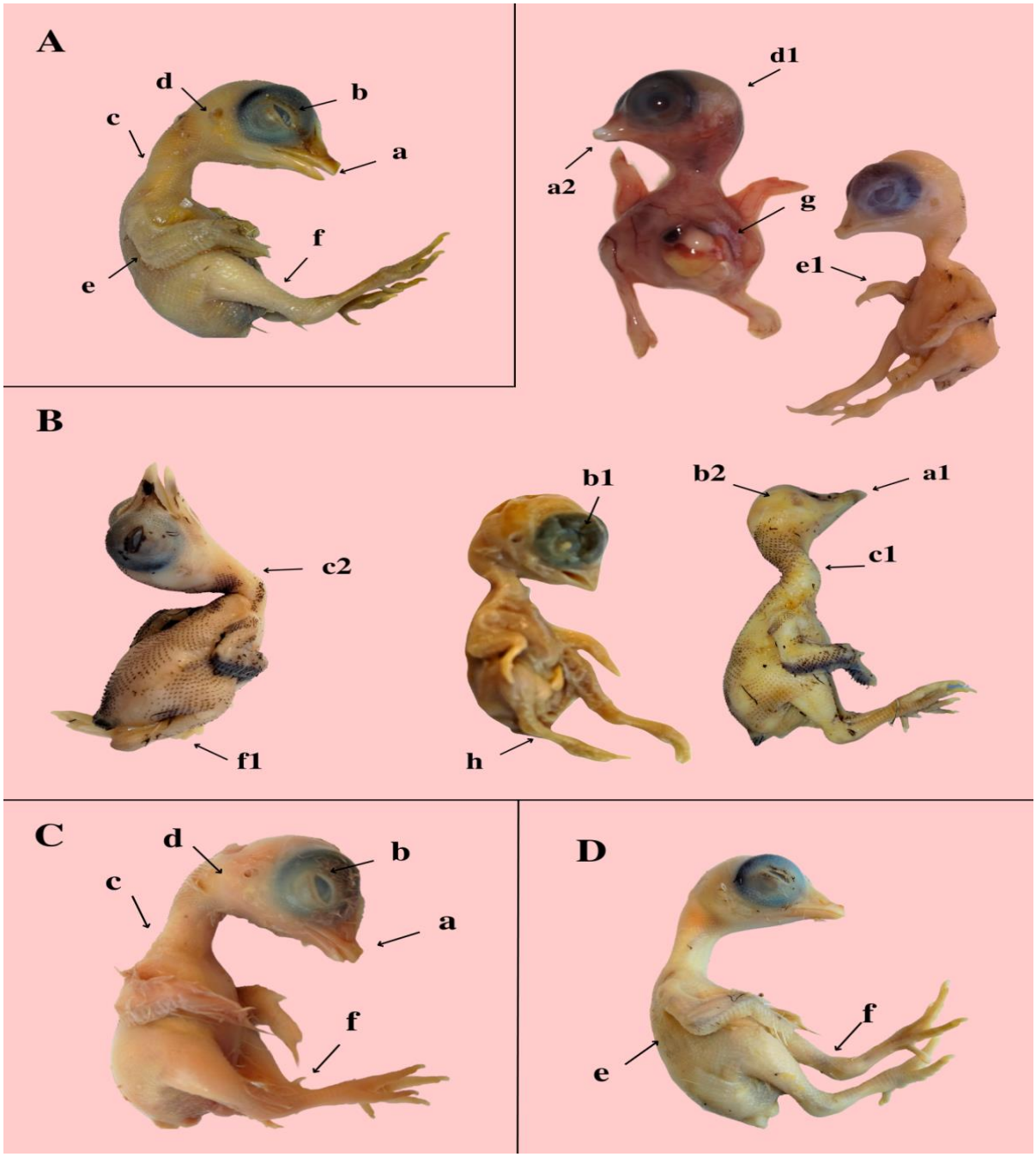

Fig. 1. **A** Control, **B** OxyG, **C** B6G, **D** OxyB6, **a:** Normal beak **a1:**Mandible enlargement **a2**: reduction of beak **b:**Normal eye development with eyelids **b1:** Embryonic cataract **b2:** Anophthalmia **c:** Normal neck **c1:** Skewed neck **c2:**Reduced neck **d:** External auditary meatus **d1:** Anotia **e :**Normal growth fore-limbs **e1:** fore-limbs reduction **f:** Normal growth of hind-limbs **f1;** Reduced hind-limbs **g:** Hemorrhgia h: Muscular destrophy

significant impact of OXY exposure on embryonic development (fig. 1). Embryos recovered from OxyB6 group displayed milder anomalies as compared to OxyG, including improved body weight and size, improved beak and bone development, absence of neck skewing, limb torsion or phocomelia.

Morphometric Results

The mean embryo weight was significantly increased ($p \leq 0.05$) in both B6G and OxyB6 groups as compared to OxyG. Mean crown ramp length, as well as fronto-occipital and biparietal measurements, were significantly ($p \leq 0.05$) lower in OxyG as compared to the control and B6G groups. The mean length of the left shank, anti-brachium, and brachium in OxyG groups were significantly lowered as compared to the OxyB6 group. Similarly decreased mean length was noted for the right shank, anti-brachium, and brachium in OxyG as compared to the control. However, there was no significant difference ($p>0.05$) in the length of the phalanges between the groups.

Table 1: Variations in 14-days chick embryo morphometric measures. Data shown as Mean ± SEM, Statistical analysis was by two-way ANOVA. a, b, c, d, e: Any two groups not sharing a lowercase letter differed significantly *(p < 0.05)* from each other (post hoc analysis)[1]

Morphometric parameters	Control	OXY	B6	OXY&B6
Mean weight of embryo (g)*§	10.70±0.80^{a}	5.55±0.86^{c}	9.88±0.82^{b}	6.53±0.77^{ac}
Mean length of crown-rump (mm)*§§	52.74±1.70^{a}	39.77±4.46^{c}	53.72±1.18^{a}	43.42±4.03^{b}
Mean length of fronto-occipital (mm)*§	16.16±0.66^{c}	12.39±0.71^{a}	14.60±0.68^{b}	14.02±0.63^{b}
Mean distance of biparietal (mm)*§	12.65±0.52^{a}	9.09±0.56^{c}	13.95±0.54^{ab}	10.88±0.51^{a}
Mean eye width (mm)*§	8.86±0.37^{a}	8.84±0.40^{ab}	9.31±0.38^{b}	9.73±0.36^{abc}
Mean eye length (mm)*§	9.41±0.38^{a}	8.84±0.41^{bc}	9.79±0.39^{ab}	10.61±0.37^{a}
Mean circumference of eye (mm)*§	30.12±0.94^{a}	27.16±1.01^{a}	31.97±0.97^{b}	30.87±0.91^{b}
Mean length of maxilla (mm)*§	9.82±0.46^{a}	6.86±0.49^{c}	8.92±0.60^{b}	8.80±0.47^{b}
Mean length of mandible (mm)*§	8.59±0.58^{a}	5.30±0.63^{c}	8.20±0.60^{b}	8.31±0.57^{b}
Mean length of left anti-brachium (mm)*§	9.62±0.70^{a}	6.30±0.76^{c}	9.09±0.72^{b}	9.76±0.68^{b}
Mean length of right anti-brachium (mm)*§	9.66±0.54^{a}	6.55±0.58^{a}	9.14±0.55^{b}	9.42±0.51^{b}
Mean length of left brachium (mm)*§	9.19±0.63^{a}	6.73±0.67^{a}	9.17±0.64^{b}	9.16±0.60^{b}
Mean length of right brachium (mm)*§	9.22±0.62^{a}	6.78±0.68^{a}	9.19±0.65^{b}	9.12±0.61^{b}
Mean length of left shank (mm)*§§	9.75±0.57^{a}	7.48±0.62^{c}	9.75±0.58^{b}	9.42±0.56^{b}
Mean length of right shank (mm)*§	10.04±0.53^{a}	7.56±0.57^{b}	9.77±0.55^{b}	9.58±0.51^{b}
Mean length of left thumb (mm)§§	4.00±0.46^{a}	3.13±0.58^{b}	4.00±0.13^{a}	3.20±0.50^{b}
Mean length of right thumb (mm)§§	4.31±0.38^{a}	3.08±0.83^{c}	5.16±0.11^{ab}	3.42±0.51^{bc}
Mean length of left index finger (mm)*§	7.10±0.56^{a}	4.73±0.60^{c}	7.71±0.57^{b}	6.91±0.54^{b}
Mean length of right index finger (mm)*§	7.10±0.54^{a}	4.78±0.58^{c}	7.75±0.56^{b}	6.10±0.52^{b}
Mean length of left middle finger (mm)*§	10.32±0.79^{a}	6.72±1.89^{c}	10.44±0.38^{b}	7.58±1.79^{b}
Mean length of right middle finger (mm)§§	9.42±0.62^{a}	6.54±1.78^{b}	10.17±0.30^{b}	7.58±0.98^{b}
Mean length of left little finger (mm)*§	7.06±0.50^{a}	4.82±0.53^{c}	7.17±0.51^{ab}	6.35±0.48^{b}
Mean length of right little finger (mm)*§§	7.19±0.52^{b}	4.68±0.56^{c}	6.99±0.54^{b}	6.03±0.50^{bc}

[1] Values are Mean ± SEM (n = 40) Groups sharing the different letter (a, b, c) are significantly different (P < 0.05).

DISCUSSION

Continuous in ovo herbicide exposure leads to embryotoxicity, raising concerns about environmental field hazards. Minor environmental variations can disrupt embryo development. This study assessed teratogenic effects of OXY and protective effects of Vitamin B6 against damaging effects of OXY exposure on chick embryos. OXY induces oxidative stress by generating reactive oxygen species which hinders body growth, leading to reduced forelimb and hindlimb development and accumulation of toxic metabolites [18]. It results in an enhanced level of embryonic mortality and a range of teratogenic abnormalities, including impaired development, beak reduction, muscular dystrophy, hemorrhagic spots, feather loss, limb defects, and cataracts. These effects are parallel to the findings of a study on teratogenic effects of lambda cyhalothrin on chick embryo [19]. OXY exposure also resulted in irregular eye development, neck misalignment, incomplete bone ossification and embryonic cataracts. These abnormalities may be due to diminished levels of respective proteins synthesis. Protein synthetic machinery impairment may occur due to toxicity and necrosis of cells[20].

Previous research has observed similar embryonic malformations and skeletal deformities resulting from in ovo exposure to cypermethrin and chlorpyrifos. Cypermethrin's teratogenic effects include skeletal deformities such as shortened body length, microcephaly, and micromelia [4]. A similar study on

chick embryo development demonstrated that bifenthrin has teratogenic potential, affecting the liver and kidneys [21].

Vitamin B6 plays a protective role, enhancing growth performance by countering oxyfluorfen-induced protein depletion. Pyridoxine treatment alone boosts embryo body weight due to increased protein retention, underscoring its physiological importance [22, 23]. Recent scientific discoveries reveal a novel protective role for vitamin B6, with antioxidant and anti-inflammatory properties that suppress oxidative stress and inflammation [24]. Vitamin B6, particularly pyridoxine, acts as a scavenger of reactive oxygen species (ROS) 25, potentially reducing lipid peroxide levels and superoxide radical concentrations in vascular-endothelial cells induced by hydrogen peroxide (H_2O_2) [26]. Additionally, vitamin B6 can regulate cellular calcium influx through purinergic mechanisms, including ATP-mediated and voltage-mediated pathways. This regulation suggests a role in maintaining calcium concentration for bone growth [27].

Vitamin B_6's protective effect in this study is probably multifaceted. Pyridoxine is a cofactor in many enzymatic processes that are involved in the creation of neurotransmitters and the metabolism of amino acids [28]. It promotes healthy development and protein production. Reactive oxygen species scavenging and lipid peroxidation inhibition are antioxidant properties. It may help lessen the effects of oxidative stress on developmental changes [29]. Both mechanisms can contribute to the ameliorative potential of the vitamin B6.

In ovo pyridoxine injections improves the development of chick embryo as referred by the study of turkey poults [17]. Vitamin B6 supplementation has been shown to enhance immune function in both humans and animals [30, 31] and improve antibody production while enhancing the interaction between cytokines and chemokines [32]. Oxyfluorfen reduces hemoglobin concentration, while pyridoxine enhances red blood cells and hemoglobin [16]. Additionally, vitamin B6 appears to serve a neuroprotective role [14] and plays a crucial part in lowering plasma homocysteine levels, an important factor in preventing cardiovascular diseases [32]. It acts as a cofactor in inflammatory pathways [30]. Furthermore, with the assistance of vitamin B6, ethylene glycol can be metabolized through non-toxic pathways leading to glycine, as opposed to harmful pathways that produce toxic metabolites [33].

The study highlights the teratogenic impacts of oxyflourfen and ameliorative potential of vitamin B6. However, the study has some limitations. Results are based just on morphometric data, which may ignore molecular or functional aspects. The study has single species and developmental stage. Understanding of harmful levels and possible post-hatch consequences is limited in the absence of a dose-response study and long-term follow-up. Furthermore, natural exposure pathways might not be entirely replicated by in ovo administration.

To summarize, pyridoxine (vitamin B6) acts therapeutically to counteract the toxic effects of oxyfluorfen, primarily by alleviating oxidative stress in differentiating tissues. This results in improved embryo growth, increased weight, and enhanced bone length.

CONCLUSIONS

The findings of the study report the teratogenic effects of herbicides such as oxyflourfen on developing embryo. These effects can be treated with antioxidants such as vitamin B6. Pyridoxine may be able to reduce the teratogenic effects of herbicides throughout the development of the embryo by supporting protein synthesis and providing antioxidant defense. To clarify the main mechanism of pyridoxine activity, future research should combine morphometric analysis with molecular and biochemical testing. It should also investigate its potential application as a protective agent against developmental toxicity in agricultural and environmental settings.

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DISCLOSURE OF FINANCIAL AND NON-FINANCIAL RELATIONSHIPS AND ACTIVITIES AND CONFLICTS OF INTEREST

None.

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