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Studies On Synergistic Effects of Lambda-Cyhalothrin and Methylcobalamin (Vitamin B12) On Development Of Chick Embryo

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ABSTRACT

Purpose: Chicken embryos serve as a prominent model in embryonic development research. The present study evaluates the developmental impact of Lambda-Cyhalothrin and Methylcobalamin (Vitamin B12) on *Gallus domisticus* chick embryos.

Methods: 250 fertilized eggs of *Gallus domesticus* were distributed into five groups: i) Control group (CG): incubated without any treatment ii) Vehicle Control group (VC): injected with 0.1mL of a 5% DMSO solution in water iii) Lambda-Cyhalothrin group (LCH): injected with 0.1mL of 0.01mg/kg LCH solution in 5% DMSO iv) Vitamin B12 group (B12): injected with 0.1mL of 0.1mg/kg vitamin B12 solution in 5% DMSO v) Lambda-Cyhalothrin group + Vitamin B12 (LCH+B12): injected with 0.1mL of 0.01 mg/kg LCH 0.1mg/kg vitamin B12 solution. Treatment was given on zero day and embryos were recovered on 14th day after incubation to check embryonic development.

Results: Morphological observations indicated that both the CG and VC groups displayed normal development. In contrast, the LCH and LCH+B12 group exhibited adverse effects, which included embryonic cataracts, neck deformities, muscular dystrophy, and a higher rate of embryo mortality when compared to the CG and VC groups. Moreover, LCH+B12 group also showed severe synergistic embryonic toxicity, characterized by halted development, foul odor, sulfur contents, blackish discoloration and teratogenic signs including embryonic cataract, evisceration, halted neck, hemorrhage spot and reduced limb size.

Conclusion: Type-II fluoridated pyrethroid LCH is highly toxic to chick development. Study revealed that Vitamin B12 alone does not interfere with normal development however its co-treatment with LCH can aggravate toxic potentials of LCH for embryonic development in the chick.

Keywords: *Gallus domesticus*, Lambda-Cyhalothrin, Vitamin B12, Methylcobalamin, Teratology

INTRODUCTION

LCH is one of the most extensively employed Type II fluorinated pyrethroid[1-3] insecticides widely used for controlling pests in various instances, including agriculture, public health homes and gardens. [4,5] Due to efficacy and toxicity, LCH is prioritized over organochlorines, organophosphates, and carbamates. [6] The lipophilic nature of LCH facilitates its rapid absorption into biological membranes[7], leading to neurotoxicity, paralysis, and even death. [8-10] Its major target is the neuromuscular junction that controls muscle contraction and movement of body parts.[11] LCH enhances the production of reactive oxygen species and mutagenic compounds thus promoting inflammation and atherogenic activity and development abnormalities.[12,13] In mice, LCH has been reported to cause embryo malformations, specifically characterized by reduced bone ossification in certain skeleton parts.[14] Similarly in rats prenatal exposure to LCH has been found to effect memory formation in developing embryo.[15]

Vitamin B12, a water-soluble vitamin, having molecular formula $C_{63}H_{88}CoN_{14}O_{14}P$ is an essential cofactor/coenzyme[16], plays a pivotal role in metabolizing fatty acids, carbohydrates, nucleic acids, and maintaining cellular function. [17, 18] It is widely used in routine medication as a therapeutic agent against various types of illness.[19] Vitamin B12 is generally considered safe; however, there have been some reports of adverse consequences in the medical literature, [20] including skin and central nervous system toxicity.[21]

This study aims to elucidate the teratogenic consequences of LCH exposure on chick embryos and to evaluate the role of vitamin B12 supplementation against LCH-induced teratogenicity.

MATERIAL AND METHODS

Experimental Groups

250 freshly laid fertilized eggs of golden black common chickens, were swiftly gathered within 24 hours from neighboring villages in Sargodha city.Eggs within a weight range of 34 to 38g were randomly distributed into five groups of 50 each, based on the injected solutions for experimentation.

i) Control Group (CG): Incubated without any treatment or dosage

ii) Vehicle Control group (VC): Injected with 0.1mL of 5% DMSO (due to its high solubility and low toxicity) solution in water. This group ensures observed effects are solely attributable to the toxicant.

iii) Lambda-Cyhalothrin group (LCH): Injected with 0.1mL of 0.01 mg/kg LCH solution in 5% DMSO.

iv) Vitamin B12 group (B12): Injected with 0.1 mL of 0.1mg/kg vitamin B12 solution in 5% DMSO.

v) Lambda-Cyhalothrin group + Vitamin B12 (LCH+B12): Injected with 0.1mL of 0.01mg/kg LCH+0.1mg/kg vitamin B12 solution in 5% DMSO.

Dose administration

To eliminate any surface germs, eggs were washed, labeled, and sterilized with 70% alcohol using cotton swabs, before air-drying. Egg shells were softened with HCL (1 drop was administered using a 1mL syringe), this facilitate the injection process, then they were laid horizontally for around 4-5 minutes to allow the embryos to rise to the top that minimizes the risk of inadvertently puncturing the embryo. The eggs were positioned horizontally for 5-6 minutes to allow the yolk to ascend to the top. Using sterilized syringes, a dose was then injected into the yolk sac of the fertilized eggs through a window, the window was properly sealed after the dosage administration to prevent contimination. Prior to placing the eggs in the incubator, the weight of each egg was measured to ensure uniformity and to assess egg quality using an automated weight balance.

Incubation

Eggs were incubated for 14 days in the Nanchang Vena egg incubator, with a capacity of 48 eggs, having precise controls and backup systems to maintain a constant 37.5°C temperature and 60% humidity, with water refill intervals adjusted every 48 hours in winter and 24 hours in summer, ensured optimal conditions. Daily candling was done,for monitoring egg fertility, embryo development and eggs showing stuttered or retarded development, lack of vascularization and absence of embryoic development_were systematically removed from the incubator.

Recoveries

On 14th day, the embryos were recovered because this developmental stage corresponds to significant organogenesis. The eggs were weighed, and by using forceps to handle tissues and scissors to make precise cuts, delicately removed a spherical portion of the shell from its broader side. Due to the lighter weight of the embryo compared to the yolk, it floated above the yolk after the removal of the shell piece. Subsequently, the egg contents, including the embryo, were transferred to a petri dish filled with a saline solution that cleanse the embryos and preserves hydration levels. The embryo was carefully isolated from the yolk using sterilized forceps and a camel hair brush, employing aseptic techniques to maintain purity and ensured gentle handling to prevent damage.A fixative solution comprising 90mL of alcohol and 10mL of saturated formaldehyde was utilized to preserve the extracted embryos, as it effectively immobilizes cellular structures and prevents decay. The embryos were immersed in this mixture for 48 hours to ensure thorough penetration, maximizing preservation quality and maintaining tissue integrity for accurate examination.

Morphometric measurement & Data analysis

Morphological measurements of all groups including embryo weight, crown-ramp length, front-occipital length, biparietal distance, eye length, forelimb and hindlimb length and all digit lengths of hindlimb were taken by using vernier caliper with no zero error.

The morphometric data was then analyzed using ANCOVA (Analysis of Covariance) and ANOVA (Analysis of Variance) tests in IBM SPSS Statistics 22 software. ANOVA was employed to analyze crown-ramp length, while ANCOVA was utilized for all other parameters, treating crown-ramp length as a covariate. These statistical tests were utilized to assess the differences and relationships between the measured variables while controlling for potential covariates.

RESULTS

Morphological Results

The CG and VC group embryos exhibited normal morphological characteristics, including the development of forelimbs and hind limbs, beak and eye development, down development, and a normal increase in size. LCH exposure resulted in various abnormalities, with the most specifically being a reduction in embryo size, embryonic cataracts, and anophthalmia. Additionally, other noticeable deformities included reduced beak size or anathia, a skewed spine, hemorrhagic spots on the body, and muscular dystrophy. Furthermore, appendicular defects were observed, such as meromelia and amelia of forelimbs, along with hindlimb deformities like phocomelia, torted shank, and abnormally developed or absent digits. Chick embryos treated with only vitamin B12 showed similar growth, weight, and general development patterns as observed in the CG and VC group (Figure 1).

In contrast to the anticipated outcome, the introduction of vitamin B12 as part of the treatment regimen to counteract the adverse effects of LCH yielded unexpected results in the LCH+B12 group. Instead of providing a protective effect, this group exhibited an increased incidence of abnormalities and teratogenic characteristics . The majority of the LCH+B12-treated eggs showed halted embryonic development, along with a blackish sulfur content and a strong odor, indicating significant deterioration and heightened embryo mortality (Figure 1).

The embryos in the LCH+B12 group demonstrated reduced weight, along with the development of embryonic cataracts, evisceration, muscular dystrophy, and diminished limb size and these sysmtoms was more severe than LCH group. Furthermore, this group showed observable differences in measurements like as front-occipital lengths, biparietal distances, beak

lengths, eye circumferences, and appendicular lengths. These variations in morphometric parameters indicate a substantial influence of the combined treatment on the overall development and structural characteristics of the embryos. These results are illustrated visually in Figure 1, which provides a clear representation of the identified anomalies and disparities among the LCH+B12 group (Figure 1).

The detrimental impact of the LCH+B12 treatment on embryo weight, developmental abnormalities, and various body parameters raises concerns about its safety and effectiveness in preventing LCH-induced adverse effects. Further investigation is required to elucidate the underlying mechanisms responsible for these unexpected outcomes and to reassess the potential therapeutic applications of Vitamin B12 in LCH management (Figure 1).

Morphometric Results

Morphometric results indicate that the mean weight of the embryos demonstrated a decrease in weight in both the LCH and LCH+B12 groups compared to the CG and VC (vehicle control) group. Differences in morphometric parameters among experimental groups imply potential teratogenic effects of LCH and LCH+B12. Statistical analysis did not reveal a significant difference ($p>0.05$) between the LCH and LCH+B12 groups. In terms of other parameters, including crown length, front-occipital length, Bipartial distance, and eye circumference, a significant decrease ($p<0.05$) was observed in both the LCH and LCH+B12 groups compared to the CG, VC and Vitamin B12 groups. Conversely, for beak length the mean values of the LCH and LCH+B12 group were significantly lower in comparison to the CG and VC group, while the Vitamin B12 group exhibited significantly higher values ($p<0.05$) than the control and VC group. Similarly, significant decreases ($p<0.05$) were noticed in the LCH and LCH+B12 groups compared to the other groups for shank, brachium and anti-brachium length (Table 1).

Furthermore, the mean values of the index left finger, little finger, and thumb showed a slight significant decline ($p<0.05$) in the LCH, and LCH+B12 group compared to Vitamin B12, and VC group and CG group. The values of the middle finger in the VC group were significantly higher than the CG and B12 group (Table1).

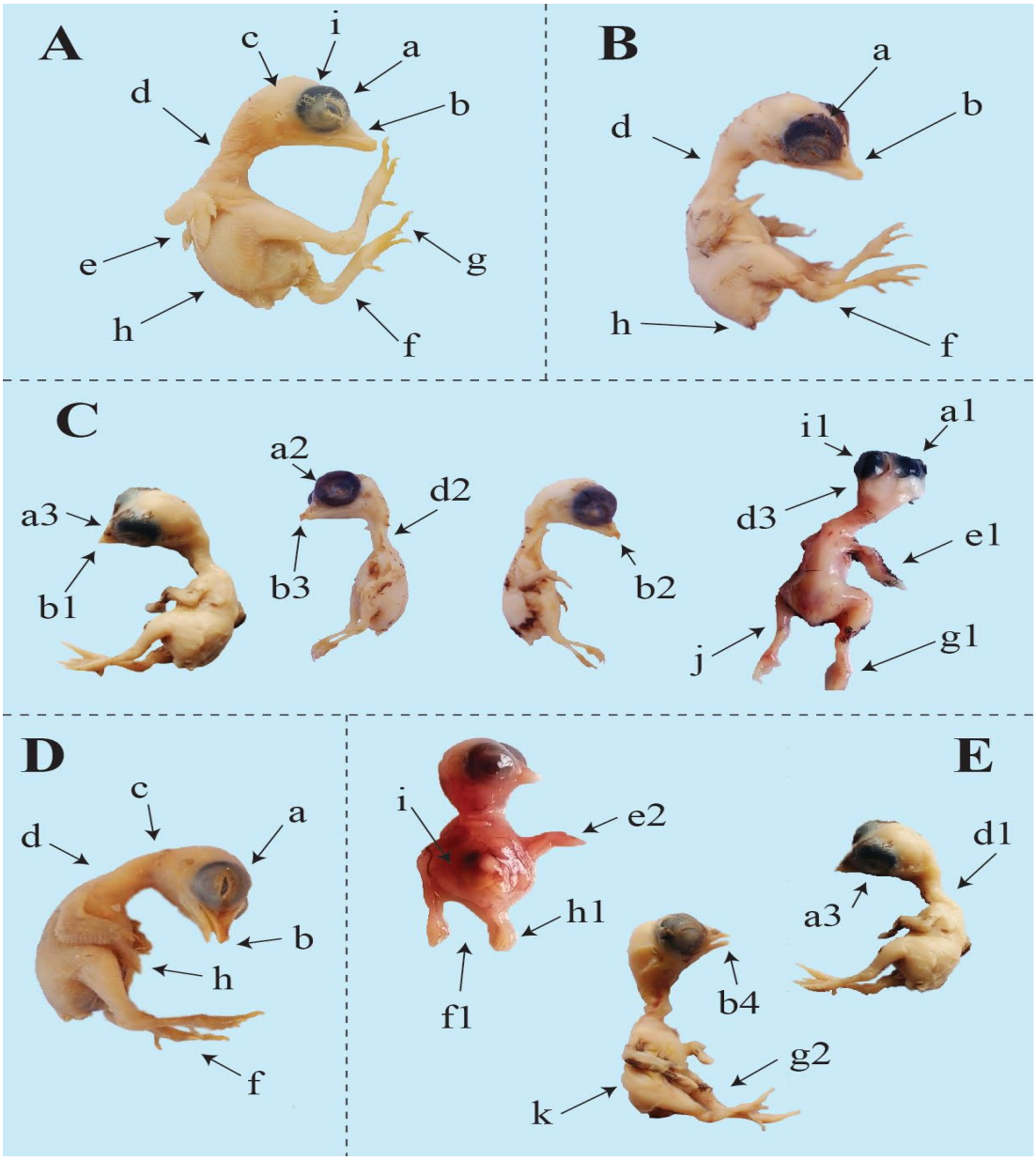

Figure 1. (A) Control, (B) VC (C) LCH, (D) B12, (E) LCH+B12,a: Normal eye **a1:** Anophthalmia, **a2:** Bulging eye **: a3:** Embryonic cataract **b:** Normal beak **b1:** reduce beak or anathia **b2:** reduce lower beak **b3:**elongated maxilla **b4:** permeant open beak **c:** Normal Auditory canal **d**: normal neck development **d1** skewed neck **: d2:** elongated neck **d3:** Halted neck **e**: normal forelimb **e1:** forelimb meromelia **e2:** forelimb edema **f:** normal hind limb **f1:** torted Shank **g:** hind limb digits normal formation **g1**: Phocomelia **g2:** reduce hind limb **h:** normal down feather formation **h1:** no down feather development **i:** normal head development **i1:** Distorted head **I:** evisceration **J:** torted shank **K:** muscular atrophy

Table 1: Morphometric analysis of 14-day chick embryo.

Morphometric Parameters	⁑Mean +SEM				
	Control	Vehicle Control	LCH	B12	LCH+B12
Mean Weight of embryo(g)*§	7.47±0.86^{bc}	7.96±0.74^{c}	5.50±0.74^{a}	7.01±0.75^{b}	5.54± 0.71^{a}
Mean Crown- ramp length (mm)**§§ €	41.57±0.71^{bc}	40.05±1.06^{abc}	39.02±1.25^{abc}	40.75±1.80^{b}	37.70±1.55^{a}
Mean Fronto-occipital length (mm)*§	13.28±0.61^{c}	14.94±0.61^{c}	2.19±0.711^{ab}	13.20±0.58^{ab}	13.08±0.62^{a}
Mean Bipartial distance (mm)*§	11.88±0.61^{b}	12.75±0.42^{c}	11.29±0.53^{b}	12.19±0.53^{b}	10.77±0.50^{a}
Mean eye length (mm)*§	9.56±0.47^{b}	10.00±0.41^{c}	8.72±0.41^{a}	9.60±0.42^{b}	8.56±0.39^{a}
Mean eye width (mm)*§	9.23±0.37^{c}	9.86±0.32^{c}	8.66±0.33^{b}	8.37±0.33^{a}	8.18±0.31^{a}
Mean eye circumference (mm)*§	29.45±0.94^{a}	31.19±0.81^{a}	27.30±0.81^{a}	28.19±0.82^{a}	26.60±0.77^{a}
Mean Beak length (Lower) (mm)***§	9.18±1.01^{bc}	9.20 ±0.87^{bc}	8.29 ±0.87^{a}	10.20±0.88^{b}	8.28 ±0.83^{a}
Mean Beak length (Upper) (mm)*§	9.75±0.79^{c}	9.22±0.68^{c}	7.64±0.68^{a}	9.31±0.69^{b}	7.50±0.65^{a}
Mean Brachium length (Left) (mm)**§	8.60±0.63^{b}	9.16±0.55^{c}	7.05±0.55^{ab}	8.12±0.55^{b}	6.17±0.52^{a}
Mean Brachium length (right) (mm) *§	7.75±0.57^{b}	8.84±0.49^{b}	7.16±0.50^{a}	7.97±0.50^{b}	7.13±0.47^{a}
Mean Anti-brachium length (left(mm)*§	7.32±0.82^{c}	9.80±0.71^{bc}	7.49±0.71^{ab}	9.79±0.72^{b}	7.38±0.68^{a}
Mean Anti-brachium length (right) (mm)*§	7.76±0.63^{a}	7.93±0.55^{b}	5.04±0.55^{a}	7.98±0.55^{b}	5.03±0.52^{a}
Mean Shank length (left) (mm)***§	10.19±0.81^{c}	10.34±0.70^{c}	7.78±0.71^{ab}	8.26±0.71^{ab}	7.76±0.67^{a}
Mean Shank right length (mm)**§	9.51±0.80^{c}	10.30±0.69^{c}	8.81±0.70^{ab}	8.73±0.70^{ab}	9.52±0.66^{a}
Mean thumb length (left) (mm)**§	2.62±0.32^{c}	3.57±0.27^{b}	2.86±0.28^{a}	3.43±0.28^{b}	2.81±0.26^{a}
Mean thumb length (right) (mm)*§	2.71±0.35^{a}	3.27±0.30^{b}	2.46±0.30^{a}	3.31±0.31^{b}	2.32±0.29^{a}
Mean Index finger length (left) (mm) **§	4.46±0.48^{a}	5.66±0.41^{b}	4.66±0.42^{a}	4.79±0.42^{a}	4.50±0.40^{a}
Mean index finger right length (mm)*§	4.44±0.51^{b}	5.45±0.44^{b}	3.80±0.44^{a}	5.95±0.44^{b}	3.78±0.42^{a}
Mean middle finger length (left) (mm)*§	5.64±0.71^{a}	8.28±0.62^{c}	6.18±0.62^{a}	5.64±0.63^{b}	6.09±0.59^{a}
Mean middle finger length (right) (mm)*§	5.45±0.79^{a}	7.76±0.69^{a}	6.04±0.69^{a}	6.73±0.70^{b}	5.77±0.66^{a}
Mean little finger length (left) (mm)*§	4.34±0.41^{b}	5.92±0.35^{b}	3.55±0.35^{a}	5.13±0.36^{b}	3.56±0.34^{a}
Mean little finger length (right) (mm)***§	4.59±0.54^{c}	5.61±0.47^{b}	3.93±0.47^{a}	5.39±0.48^{ab}	3.82±0.45^{a}

⁑Mean values ± SEM (Standard Error of the Means); Mean values were calculated by averaging measurements within each experimental group, while the standard error of the mean (SEM) indicates the precision of these calculated means. *: P≤ 0.05, **: P≤ 0.001 ***: P≤ 0.0001, §: Analyzed by ANCOVA (**Analysis of CO-variance)**; §§: Analyzed by ANOVA (Analysis of Variance**)**employing Crown-ramp length as Co-variant, ^{abc}Groups in the table not sharing a common upper-case letter signify statistically significant differences between them.

DISCUSSION

Lambda-cyhalothrin has been documented to exhibit significant levels of toxicity. This study was designed to elucidate the teratogenic abnormalities caused by LCH in chick embryos as a model organism. Current findings also revealed that exposure to LCH alone as well as in combination with vitamin B12 had detrimental effects on the embryos.

The study's revelations highlighted the susceptibility of developing embryos to manifest diverse morphological anomalies in response to exposure to LCH. These anomalies encompass limbic abnormalities, impaired muscle growth, embryonic cataracts, reduced developmental progression, and, in severe cases, mortality. Furthermore, the growth of claws and down feathers was negatively impacted by LCH exposure. Multiple fluoridations in LCH molecule may be the cause of its teratogenic potentials[22,23] as the results of the present study are consistent with our previous study on in-ovo exposure of bifenthrin and oxyflourifen fluoridated pyrethroid insecticide and with cypermethrin.[24-28]

Results align with previous research, which has demonstrated that exposure to LCH leads to protein depletion and necrosis of cellular function. Consequently, affected tissues experience impaired differentiation and metabolic incapacity to degrade the insecticide and eliminate its byproducts. Moreover, they have to counteract the reactive oxygen species generated as a result of insecticide exposure.[29, 30] It documented analogous deformities in chick embryos exposed to LCH. Limited data exist regarding the impact of LCH on birds, and there is insufficient evidence of LCH accumulation in avian tissues or eggs.[31] So, this study explores LCH effects and accumulation in avian tissues/eggs.

In this study, the decision to use vitamin B12 was based on understanding its critical role in various physiological processes, embryonic development and neurological processes, suggesting its potential to mitigate the adverse effects of teratogens. Previous researches has demonstrated its involvement in DNA synthesis, cell division and nervous system development.[32] Hence, the hypothesis was formulated that vitamin B12 supplementation might positively impact chick embryo development by reducing developmental abnormalities against the teratogenic consequences associated with LCH exposure. However, actual findings revealed that vitamin B12 supplementation along with LCH could lead to adverse concequences such as impaired embryo development. Moreover, studies have indicated that vitamin B12 may contribute to fetal developmental abnormalities during pregnency.[33]

The unexpected adverse results observed in the combination of LCH and vitamin B12 suggest the presence of a toxic synergistic effect. Contrary to the hypothesis, vitamin B12 did not provide the anticipated protective effects or act as an antioxidant to mitigate the harmful effects of LCH. Instead, its use in combination with LCH worsened developmental abnormalities. As a consequence of this toxic synergistic interaction, a considerable number of eggs exhibited developmental failure, characterized by a malodorous odor and the presence of blackish soot. Moreover, the affected embryos displayed various teratogenic manifestations, including cataracts, abnormal beak development, reduced weight, torticollis, evisceration, muscular dystrophy, limb abnormalities, and embryonic edema. Study revealed that the limited expression of genes involved in vitamin B12 metabolism during embryogenesis suggests that disruptions in vitamin B12 metabolism and related proteins can lead to developmental abnormalities throughout embryonic development.[34]

Additionally, vitamin B12's role in stimulating muscles and regulating contractile activity may contribute to disturbances in muscular development and further contribute to embryonic anomalies.[35] While it is generally considered safe, there have been reports of negative consequences in certain cases including gastrointestinal, neurological, skin, and subcutaneous issues.[18]

The exact mechanisms underlying the toxic synergistic effects between LCH and vitamin B12 are not fully understood. It is plausible that the interaction between these substances interferes with essential pathways involved in embryonic development. The unexpected exacerbation of developmental abnormalities in the LCH+B12 group indicates a complex and potentially harmful interaction between LCH and vitamin B12. These findings emphasize the need for caution when considering vitamin B12 supplementation in areas where LCH is used as an insecticide. Future research should delve into elucidating the molecular

mechanisms driving the toxic synergy between LCH and vitamin B12, examining potential biomarkers and pathways involved. Additionally, histopathological examination, gene expression analysis, and biochemical assays will be utilized to better understand the effects of LCH and B12 on embryo development and morphology.

CONCLUSIONS

This study reveals the harmful synergistic effects of administering vitamin B12 with LCH on chick embryo development. The findings indicate a substantial risk of inducing teratogenic defects in avian populations and suggest potential risks for human abnormalities in regions with insecticide exposure. Caution should be exercised regarding the use of vitamin B12 in areas where LCH is employed as an insecticide, moreover policymakers should enforce strict regulations on LCH use, promote safer alternatives, and educate the public on potential risks of B12 Suplimentatation where LCH is prevalent.

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CONFLICT OF INTERESTS

None

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