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Protective Effect of Vitamin C Against the Paraquat-Mediated Toxic Effects on Different Morphometric and Biochemical Parameters in Chick Embryos of *Gallus Domesticus*

Subita Choudhary[1], Ankita Kanwar[2], Priyanka Mathur[2], Mahesh Kumar Saini[3], Preeti Gupta*[1],

1. Department of Zoology, LBS Govt. College Kotputli, Kotputli-Behror
2. Department of Zoology, IIS deemed to be University, Jaipur
3. Department of Blue Economy, iCED, Jaipur

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ABSTRACT:

Paraquat is a toxic herbicide that controls weeds but causes environmental harm and biodiversity loss due to prolonged exposure. Its toxicity results from reactive oxygen species (ROS) production, leading to oxidative stress and cellular damage. Vitamin C, an essential nutrient and potent antioxidant, helps to protect cells from free radical-induced damage. This work investigates the efficiency of vitamin C in reducing or eliminating the harmful effects of Paraquat on various morphometric and Biochemical parameters in chick embryos of Gallus domesticus. A total of 120 fertilized Gallus domesticus (BV Vencobb) eggs were randomly divided into four groups: Control (untreated), Sham Control (vehicle-treated with distilled water), Paraquat-treated, and Paraquat plus Vitamin C-treated group. They were incubated at 37±0.5°C with 60-70% humidity and proper ventilation. The control group consisted of untreated eggs, while the sham (vehicle) group received 0.1 ml of distilled water. The treatment group was administered paraquat (PQ) at a dose of 0.05 ml per egg, and the protective group received a combination of PQ (0.05 ml) and Vitamin C (0.1 ml) per egg on the 10th day of incubation. All substances were administered into the egg via the injection method using a sterile insulin needle.

On day 18, embryos from all groups were assessed for morphometric parameters such as mortality rate, morphometric malformations, crown-rump length, wet body weight, and biochemical parameters such as protein, cholesterol, and glycogen. Results of the present study showed that the mortality rate and the number of surviving embryos having deformities were significantly increased in the Paraquat-exposed group as compared to the control group. The crown-rump length and wet body weight showed a significant (**$p \leq 0.01$) decline in the treated groups. In addition, the tested Herbicide (PQ 0.05 ml/egg) caused a significant decrease (**$p \leq 0.01$) in Protein and Glycogen levels, with notable increases in the total cholesterol content. The vitamin C-supplemented group showed signs of recovery in maintaining the overall morphology and Biochemistry of developing chick embryos against Paraquat. The findings show that even low concentrations of the pesticide can harm embryos and threaten life. The study highlights vitamin C's protective role in reducing Paraquat's toxic effects, suggesting that supplementation may help mitigate damage to morphological and biochemical parameters

INTRODUCTION

The extensive use of pesticides, including herbicides, has raised significant global concerns regarding their potential health effects on farmers working in treated fields and the general population through food and water residues. Despite the banning of certain hazardous pesticides, many others remain widely used in various countries, with limited understanding of their full impact on both ecosystems and human health (Damalas *et al.,* 2011; Garcia *et al.,* 2012). Herbicides, while offering numerous benefits in agriculture, public health, and residential areas, are particularly valuable for improving crop yield. Their cost-effectiveness and labor-saving properties make them indispensable tools in modern farming practices. However, the ongoing risks associated with their widespread application

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necessitate a deeper examination of their effects on human health and the environment.

Paraquat (methyl viologen) is a potent and non-selective herbicide that controls weeds and protects crops. It has been thoroughly studied, regularly assessed, and comprehensively evaluated for its potential risks leading to oxidative stress and cellular damage (WHO, 2004). PQ induces oxidative stress by generating many reactive oxygen species (ROS). As a powerful ROS generator, PQ leads to cellular damage in multiple organs through mechanisms such as immune suppression, inflammation, and necrosis (Li *et. al.*, 2004; Gonzalez *et.al, 2004).* Oxidative stress (OS) occurs when reactive species overwhelm antioxidant defenses, leading to damage to lipids, proteins, and DNA, thus impacting cellular survival (Sies, 1991). The antioxidant defense system regulates the formation of ROS and protects against their harmful effects. However, when ROS levels rise excessively, the system becomes overwhelmed and unable to prevent its damage (Kıvrak *et al.,* 2017). In such cases, providing "external antioxidant supplementation to animals is essential". Antioxidants neutralize and stabilize the free radicals, preventing their harmful effects.

Vitamin C is an essential nutrient that serves as a cofactor for various enzymes and acts as a highly effective antioxidant, safeguarding cells from free radical-induced damage. Consequently, many studies have emphasized the strong antioxidant properties of vitamin C, which help to develop and strengthen bones, teeth, gums, joints, skin, and tissue repair. Furthermore, studies have shown that Vitamin C supplementation exerts a protective effect by neutralizing the free radicals produced following PQ exposure. Kothinti *et al.* (2020) induced toxicity in Male Albino Wistar Rats with a single dose of PQ and demonstrated that administering Vitamin C can effectively mitigate PQ-induced changes in body weight and hematological parameters, potentially through an antioxidant defense mechanism.

The growing poultry industry's increased pesticide use raises concerns about exposure, particularly for developing embryos, which are more vulnerable to teratogenic effects (Jelinek *et al.*1982*,* Kotwani *et al.,* 1998). The chick embryo is a valuable model for developmental toxicological studies. The present study aimed to investigate the potential embryotoxic and teratogenic effects of Paraquat on morphometric and Biochemical parameters in developing chick embryos, as well as the role of Vitamin C in mitigating PQ-induced toxicity.

MATERIALS AND METHODS

Animals

Gallus domesticus was chosen as a model to study the toxic effects of Paraquat during various stages of embryonic development. The Institutional Animal Ethics Committee (IAEC Reg. No.: 1689/PO/S/13/CCSEA) approved the animal care principles per the norms of the Committee for Control and Supervision of Experiments on Animals (CCSEA), India.

Chemicals

Paraquat, with an active ingredient of 57.15%, chemically known as *N*, *N*′-dimethyl-4,4′-bipyridinium dichloride (also known as methyl viologen), was sourced from Sumitomo Chemical India Ltd. under the trade name Avast paraquat Dichloride 24% SL Herbicide, and the final dose was decided after the LC50 calculation. Ascorbic acid was prepared by dissolving 3 g of ascorbic acid (99% pure; SD Fine-Chem Limited, India) in 100 ml of 0.9% saline solution (ascorbic acid solvent). Each 0.1 ml of this solution contains 3 mg of ascorbic acid. (Zakaria and Al-Anezi, 1996).

Administration of dose

On the 10^{th} day of incubation, a single dose was aseptically administered into the airspace of fertilized *Gallus domesticus* eggs. Treatments included 0.1 ml of distilled water, 0.05 ml of paraquat (PQ), and a combination of 0.05ml PQ with 0.1 ml of vitamin C at a 1:2 dose ratio.

Experimental Design

For the experimental investigation, 120 fertilized eggs of *Gallus domesticus* (0 day of incubation) were procured from Kewal Ramani Hatchery, Ajmer, Rajasthan, India. Eggs of similar size (61–65 g) were grouped, and all shells were sterilized with 70% ethanol to eliminate external contaminants. To maintain appropriate humidity, a 1-liter container filled with

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distilled water was placed at the bottom of the incubator. The eggs were placed horizontally on metal racks and rotated twice daily. On the 10th day, candling was performed to exclude any unfertilized eggs, after which the remaining fertilized eggs were randomly divided into four groups of 30 eggs each. Group A served as the untreated control and received no injection. Group B (sham control) was injected with 0.1 ml of distilled water. Group C was treated with 0.05 ml of paraquat (PQ), and Group D received a combined dose of 0.05 ml PQ and 0.1 ml Vitamin C at a 1:2 ratio.

All injections were administered aseptically into the air sac using a sterile insulin syringe. After treatment, eggs were sealed with paraffin wax, labeled, and incubated at 37°C with 65–70% humidity until the end of the study.

Investigated Parameters

On the 18th day of incubation, embryos were examined for mortality rate, crown-rump length (cm), wet body weight (g), and gross morphological abnormalities. Morphometric assessments included measurement of crown-rump length and evaluation of any visible defects in the chick embryo. Data were recorded for both control and treated groups for comparative analysis. Freshly excised liver tissues were weighed and processed for biochemical analysis, including the quantification of total protein, glycogen, and cholesterol. Protein content was estimated using the method of Lowry *et al.* (1951), glycogen by the method of Montgomery (1957), and total cholesterol by the Liebermann–Burchard method (Zlatkis *et al.,* 1953).

Statistical analysis

Statistical analysis was performed on all recorded parameters. Data are presented as Mean ± Standard Error of Mean (SEM). The distribution of data was assessed for normality, and comparisons between groups were made using Student's t-test for normally distributed data. The Mann–Whitney U test was employed to compare mortality rates. All statistical analyses were conducted using IBM SPSS Statistics version 22. Differences were considered statistically significant at $p \leq 0.05$ compared to the control group.

RESULTS AND OBSERVATIONS

Mortality Rate

The mortality rate was recorded at the end of experimentation, i.e., 18th day of incubation, by calculating the number of dead embryos that died normally or as a result of Paraquat administration. The number of both live and dead chick embryos, illustrated in Table 1. Minimal or no mortality was observed in the untreated and sham control groups treated with distilled water during the organogenesis phase. In contrast, a significant increase in mortality was recorded in embryos that received in ovo injections of PQ. However, co-administration of PQ with Vitamin C markedly reduced embryo mortality, indicating a protective effect of the antioxidant.

Table 1: Mortality rate in different groups of chick embryos at the 18th day of incubation

Groups	No. of eggs	No. of surviving embryos	Mortality%
A	30	30	0
B	30	29	3
C	30	20	33
D	30	24	20

Morphological observations

The morphology of representative control groups of chick embryos at the 18th day of incubation is depicted in Fig. 1A. The body was fully covered with down feathers at this stage, and the head region exhibited a well-formed beak. The eyes were normal in appearance, with fully developed eyelids. The forelimbs consisted of a normal humerus, radius, ulna, metacarpus, and digits. The hind limbs appeared normal, with scales covering the dorsal surface and keratinization extending to the base of the toenail. It included the femur, tibia-fibula, metatarsus, and digits with phalanges ending in claws, all covered in papillae on the plantar surface. The embryos in the sham control group exhibited normal structures similar to the control group (Figure 1B). However, some external malformations were observed, including failure of yolk retraction (2/29) and sparse

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body hair (1/29). Conversely, embryos treated with PQ showed marked growth retardation and several malformations (Table 2) (Figure 1C & Figure 2A). A high frequency of anophthalmia and limb deformities was observed, including flexed limbs and clinodactyly, with other malformations like a short beak, Exencephaly, and failure of yolk retraction. Moreover, 30% (13/20) of embryos showed exencephaly, and Anophthalmia was observed in 60% (18/20) of them. Vitamin C administration alongside PQ resulted in a notable reduction in PQ-induced embryotoxicity. Chick embryos in these combination groups displayed significant improvements in shape, size, and external malformations, as well as a reduction in head enlargement (Table 2, Figure 1D).

Crown-rump length

Figure 2A illustrates that the embryos in the control and sham groups exhibited the same crown-rump lengths. In contrast, the groups injected with Paraquat showed a significant (**p ≤ 0.01) reduction in body length compared to the control group. However, the injection of PQ with Vitamin C resulted in a highly significant (*p ≤ 0.05) increase in body length compared to the PQ-only group.

Wet Body Weight

In Fig. 2 B, the graph displays the changes in the embryo wet body weight in the various groups. The embryos in the control and sham groups exhibited similar body weights. A substantial and statistically significant (**p ≤ 0.01) decrease in body weight was observed in embryos injected with PQ as compared to the control group. However, administration of PQ with Vitamin C resulted in a noticeable significant (*p ≤ 0.05) improvement in body weight compared to the PQ-only group.

Biochemical investigation

A comparative study on liver biochemical contents in the sham control group showed no significant changes in protein, cholesterol, and glycogen levels compared to the control. Exposure to paraquat (PQ) (0.05 ml/egg) resulted in a significant decrease (**p ≤ 0.01) in protein and glycogen levels, while cholesterol levels significantly increased (**p ≤ 0.01) as compared to the control group. These results suggest that paraquat reduces protein and glycogen synthesis and cell viability, which aligns with its cytotoxic effects. Groups treated with a combination of paraquat and vitamin C showed significant increases (*p ≤ 0.05) in liver protein and glycogen levels, while cholesterol levels significantly decreased (*p ≤ 0.05) compared to embryos exposed to PQ alone. However, the results of the combination group did not reach the levels observed in the control group, indicating that vitamin C partially mitigated the protein-decreasing effects of paraquat (Table 4, Figure 3).

Figure 1: Photographs of 18-day-old chick embryos of the control and PQ and PQ+VitC treated groups". (A) Control embryo showing normal growth. (B) Showing sham control (C), PQ-treated embryo showing gastroschisis, anophthalmia, twisted neck, exencephaly, meromelia, sparse body hair, clinodactyly, growth retardation, and head enlargement. (D) PQ+VitC-treated embryo showing improved growth with a very low frequency of limb disorientation.

Twisted neck (Grey arrow), hematoma (Pink arrow), anophthalmia (Red arrow), blunt beak (white wavy arrow) Exencephaly (Brown arrow), gastroschisis (Black arrow), limb disorientation (Red wavy arrow), head enlargement (Wavy green arrow), SBH-sparse body hair (Wavy blue arrow), clinodactyly (curled digits) (Yellow arrow), incomplete closure of abdomen (White arrow)

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Table 2: Incidence of malformations in surviving chick embryos on the 18th day of incubation in different groups

Malformations	Groups			
	A	B	C	D
Failure of retraction of yolk	1/30	2/29	14/20	10/24
Limb deformities	0	0/29	16/20	11/24
Exencephaly	0	0/29	13/20	10/24
Anophthalmia	0	0/29	18/20	13/24
Sparse body hair	0	1/29	14/20	9/24

*Treatment given on the 10th day of incubation

Table 3 Crown rump length and wet body weight of embryos at the 18th day of incubation in different groups

Groups	Crown-rump length (CRL) (cm)	Wet weight (g)
A	10.42±.21	13.70±.020
B	10.36±.19	13.65±.018
C	8.27±.062**	10.37±.064**
D	9.41±.003*	12.21±.121*

Data are represented as Mean ±SEM. Statistical difference from the control: * significant at p≤0.05, by Student's t test. Using IBM SPSS Statistics 22 software

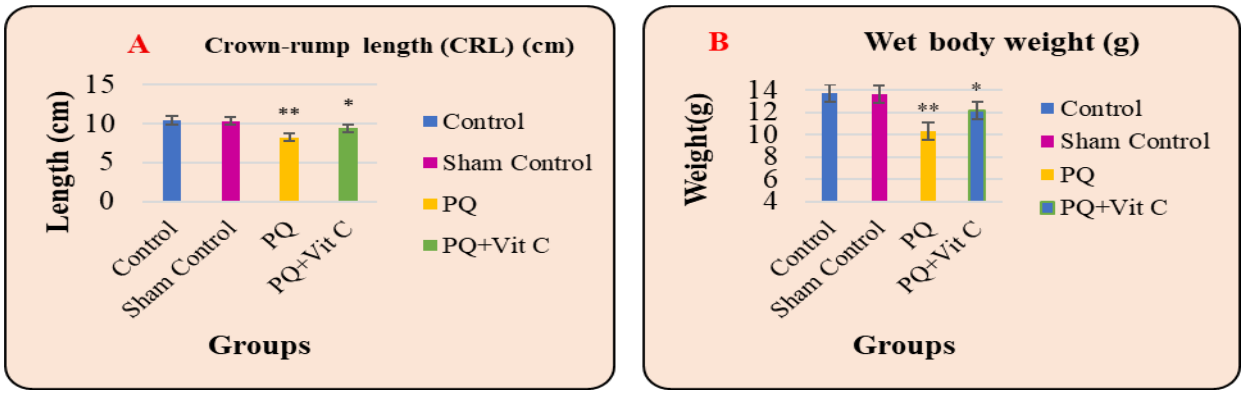

Figure 2: (A&B). Graph displaying the crown-rump length and wet body weight of 18-day-old chick embryos of various treatment groups. Data are represented as mean ±SEM. The P value in comparison to the control group is indicated by an "asterisk" (*, **). *p≤0.05(Significant), **p≤0.01(Highly Significant)

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Table 4: Showing biochemical changes in 18th-day-old chick embryos from different experimental groups at the end of the study

Groups	Biochemical Contents (mg/ g)		
	Protein	Cholesterol	Glycogen
A	4.53±0.034	3.45±0.017	2.94±0.033
B	4.49±0.081	3.40±0.024	2.91±0.042
C	3.01±0.006**	4.22±0.019**	1.82±0.024**
D	3.47±0.110*	3.72±0.013*	2.54±0.023*

Data are represented as Mean±S.E. Statistical difference from the control: * significant at p≤0.05, by Student's t test. Using IBM SPSS Statistics 22 software

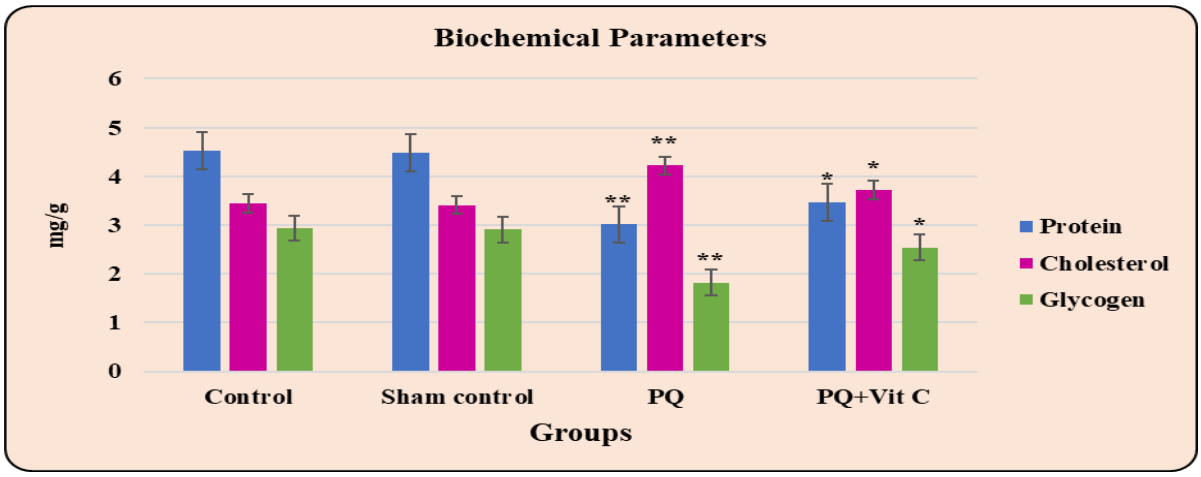

Figure 3: Graph showing the total protein, cholesterol, and glycogen of 18th-day chick embryos of different groups. Data are represented as Mean ± SEM. The P value in comparison to the control group is indicated by an "asterisk" (*, **). *p≤0.05(Significant), **p≤0.01(Highly Significant)

DISCUSSION

The present work aimed to gain insights into the defense mechanisms triggered by early chick embryos in response to oxidative stress. An acute oxidative stress condition was induced using the ROS-generating pesticide Paraquat, and its effects on the embryonic development of Gallus domesticus were assessed at both morphological and biochemical levels. Our study shows that the administration of PQ significantly disrupted embryonic development. There was a significant difference in the mortality rate between the PQ-treated group (33%) and the control (0%), sham (3%), and Vitamin C (20%) groups. PQ exposure caused extensive embryonic damage, leading to developmental delays or arrests and abnormal body pattern formation (malformations). The most common malformations observed included stunted growth, abdominal edema, gastroschisis, anophthalmia, twisted neck, exencephaly, meromelia, sparse body hair, clinodactyly, growth retardation, and head enlargement. These results are consistent with Mussi *et al.* (2010), who found that PQ treatment in C. arenarum reduced the embryos' ability to develop normally, resulting in developmental arrests and severe malformations such as tail abnormalities, abdominal edema, diminished head development, and curved dorsal structures (Mussi *et al.,* 2010). Deluao *et al.* (2022) explained that the ROS-mediated alterations in gene expression and methylation (e.g., PAX3, FOXO transcription factors) can contribute

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to neural tube defects, head malformations, and growth abnormalities (Deluao *et al.,* 2022).

Some embryos of the PQ-injected groups showed edema and exencephaly, and this is in line with Osano *et al.* (2002), who found that at 5 mg/L of PQ, all the surviving embryos of *Xenopus laevis* were Edematous and with brain deformations. The data obtained from the study revealed no effect on the crown-rump length and body weight in the sham group, while the PQ-treated groups showed a decrease in both body weight and crown-rump length when compared with the control. Also, Hoffman *et al.* (1987) examined that American kestrel *(Falco sparverius)* nestlings orally dosed daily with 25 mg/kg, or 60 mg/kg of paraquat" in distilled water showed a significant decrease in the percentage of body weight when compared with the control (Hoffman *et al.,* 1987). This growth retardation may appear due to the generation of free radicals, which results in oxidative stress and consequently metabolic disorder, which, in turn, leads to general losses of body mass (Reddy *et al.*2022).

In Liver tissue, Severe depletion of hepatic Protein was observed in embryos exposed to PQ. This is consistent with studies showing impaired albumin synthesis (reduced by ~62 in rat hepatocytes treated with PQ (Masanet *et al.,* 1988). Khan *et al.* (2003) and Sounderraj *et al.* (2011) also observed a decrease in protein levels in adult frogs following pesticide exposure. The observed decrease in protein levels may indicate physiological acclimatization to heightened energy demands in response to stress caused by PQ exposure. "Alternatively, it could reflect a mechanism for the formation of lipoproteins, which are utilized to repair damaged organelles, cells, and tissues. (Rambabu and Rao 1994; Ribeiro *et al.* 2001; Sak *et al.* 2006).

Markedly, glycogen levels also decreased with PQ exposure; these decreases accounted for a depletion of stores of this polysaccharide. This aligns with findings from Giri *et al.* (1979), who reported a marked depletion of liver glycogen in rats lasting for 48 h post-PQ injection. Glycogen plays a vital role in maintaining energy balance by serving as an internal energy reserve (Moyes and Schulte, 2010). Stressful conditions, such as pesticide exposure, which disrupt homeostasis, trigger specific physiological responses (Barton and Iwama, 1991). In response to such stress, glycogen levels are rapidly reduced to fulfill higher energy needs and facilitate the metabolic processes necessary for detoxifying pollutants (Alkahen, 1996).

The present study indicates that Paraquat (PQ) exposure increases hepatic cholesterol levels in chick embryos. Comparable results were also observed in rabbits treated with PQ, where total cholesterol was significantly increased after 7 days of PQ treatment (Hassan *et al.,* 1989). Similar findings were reported by Nouri *et al.* (2021), who observed elevated serum cholesterol and lipid levels in PQ-treated rats, likely due to oxidative stress impairing LDL receptor function and promoting cholesterol accumulation. The increase in liver cholesterol may be attributed to the inhibition of Cytochrome P450 enzymes, leading to cholesterol accumulation within cells, which raises the potential for toxicity (Stott *et al.,* 1997).

In chick embryos, co-administration of vitamin C with Paraquat (PQ) significantly reduced embryonic mortality and enhanced body mass compared to the PQ-only group. These findings align with Pinheiro *et al.* (2023), who found that optimal doses of in ovo vitamin C improved hatchling body weight while reducing mid-development mortality in chicks. In the same line, Reddy *et al (2020)* reported that vitamin C had a prominent role in ameliorating PQ-induced morphometric and gross pathological alterations in target organs of Wistar rats, possibly via an antioxidant defense mechanism. Similarly, Awadalla *et al.* (2012) demonstrated in adult rats that co-treatment with vitamin C (20 mg/kg) alongside PQ (1.5 mg/kg daily for 3 weeks) markedly attenuated PQ-induced morphological damage in liver and kidney tissues, highlighting vitamin C's protective role. Vitamin C likely improved metabolism by scavenging reactive oxygen species (ROS) and boosting antioxidant activity. As a water-soluble antioxidant, it neutralizes ROS such as superoxide, hydroxyl radicals, and hydrogen peroxide, preventing oxidative damage (Ranjbar *et al*, 2014).

Likewise, Groups treated with a combination of PQ and Vit C showed significant increases in liver protein and glycogen levels, while cholesterol levels significantly decreased compared to embryos exposed to PQ alone. Kothinti *et al.* (2020) demonstrated that supplementation with Ascorbic Acid (250 mg/kg) can

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effectively mitigate the serum biochemical changes induced by PQ (40 mg/kg), likely through the activation of an antioxidant defense mechanism.

CONCLUSION

Administration of paraquat (PQ) at a dose of 0.05 ml/egg during the organogenesis stage of developing chick embryos induced morphological malformations, along with significant reductions in total body weight and length. These effects are likely due to impaired development and incomplete formation of the embryos' defense systems. Furthermore, PQ treatment caused hepatic damage, characterized by decreased protein and glycogen content and elevated cholesterol levels in the liver. Notably, co-administration of Vitamin C demonstrated a protective effect, with some embryos showing resistance to PQ-induced damage. Embryos receiving the combination treatment exhibited improved growth parameters, indicating that Vitamin C mitigates PQ-induced developmental toxicity. Based on these findings, caution is advised when using PQ as an herbicide in agricultural practices.

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DISCLOSURES

There was no conflict of interest.

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