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RESEARCH ARTICLE

Teratogenic Effects of Thiamethoxam (a Neonicotinoid) on Development of Chick Embryo

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A B S T R A C T

The main purpose of this research project was to investigate the teratogenic effects of the thiamethoxam (TMX) on the development of chick embryo. A total of 100 fertilized eggs (72 hours old) of commercial broiler (Ross) were obtained and equally divided into five groups (A to E). The group A and B were kept as negative control and sham control, respectively. The experimental groups were administered with increasing concentrations of thiamethoxam at a dose rate of 50µg, 100µg, and 150µg each diluted in 20µl distilled water. The sham group was treated with 20µl of normal saline. After that, the eggs were incubated under standard conditions. The eggs were opened on day 20th for the evaluation of any gross anomalies and musculoskeletal deformations. The mortality rate increased gradually with the dosage of TMX. The results showed significant growth retardment in the treated chicken embryos which were further endorsed by lower body weight and reduced crown-rump length. Furthermore, the observed teratogenic effects were deformed head (exencephaly), beak agnathia, anophthalmia, scantiness of feathers, limb deformities, and failed retraction of the yolk sac. Gross and histopathological analysis of vital organs showed remarkable alterations as compared to the control group. It has been concluded that chick embryos exposed to TMX in early embryonic stages showed results in growth retardation at lower dosage, while at higher dosage marked teratogenic and histopathological effects have been recorded in liver.

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INTRODUCTION

Around the world, the use of neonicotinoids in the agricultural sector and to control ectoparasites in the veterinary sector has been increased enormously. It poses a serious threat to animals, humans, and the environment due to their toxic effects and residues in food products (Cimino *et al.*, 2017; Ahmad *et al.*, 2021). The population of various avian species is gradually declining due to the adverse effects imposed by this class of insecticides (Hallmann *et al.*, 2014; Akram *et al.*, 2021). Many studies have been published describing their toxicopathological effects on fish, mammals, and birds (Gibbons *et al.*, 2015; Gobeli *et al.*, 2017; Hasan *et al.*, 2017; Mujahid *et al.*, 2021). Poultry birds may adversely get affected by ingestion of formulated feed containing insecticide treated seeds or during exposure to spray events. Poultry feed contamination ultimately affects the quality of meat and eggs (Filazi *et al.*, 2017).

In developing countries like Pakistan, thiamethoxam (TMX) is generally employed to tackle whitefly, plant bugs, beetles, or housefly on agriculture crops of wheat, cotton, sorghum, canola, barley, and maize. Some seeds like cotton, wheat or corn seeds are being treated with thiamethoxam to control parasitic activity (Naveed *et al.*, 2010; Gul *et al.*, 2022). Ectoparasites including bugs and houseflies are controlled by different products of TMX in livestock and poultry houses (Sato *et al.*, 2019). Thiamethoxam is a synthetically available insecticide being introduced in the 1990s. TMX belongs to second generation neonicotinoids of the thianicotinyl subclass.

Other major insecticides of this group are imidacloprid, acetamiprid, clothianidin, and thiacloprid (Jeschke *et al.*, 2011). TMX is highly toxic for insects as it acts selectively on the receptors of nicotinic acetylcholine (nAChRs) located in the central nervous system. It causes the blockage of nAChRs, followed by paralysis, and ultimately death of an insect occurs. The vertebrates have a low affinity towards TMX due to differences in receptor subtype, but severe toxic effects have been reported in higher concentrations (Tomizawa and Casida, 2011).

In birds, the reported LD50 values of TMX range from 576 mg/kg to 1552 mg/kg of body weight. Higher doses of TMX cause acute toxicity in avian species or mortality may occur in some cases (Mineau and Palmer, 2013). In an epidemiological study, TMX was reported in the residual form of 0.01mg/kg in poultry meat and eggs by the European Food Safety Authority (EFSA *et al.*, 2018). Excessive usage of non-registered and substandard products of the neonicotinoids was thought to be the problematic cause (EFSA *et al.*, 2018). TMX lowered the feed consumption and impaired the liver function in laying hens. The egg production was significantly reduced, and thinness of eggshells was also observed (Gul *et al.*, 2020). Thiamethoxam in combination with some other neonicotinoids caused embryotoxicity and arrested growth in mice and rabbits. TMX had deteriorative impacts on the reproductive system of mice. It also compromised the life span of mice and rabbits when given in higher amounts (Babeľová *et al.*, 2017; Hasan *et al.*, 2017). Histopathological analysis of the liver in experimental rats treated with TMX revealed remarkable hydropic degeneration, congestion of blood vessel, focal hepatic necrosis, and phagocytic cell infiltration also observed in liver sections (El Okle *et al.*, 2016).

Clothianidin (CTD) neonicotinoid having close resemblance with TMX, proved to be toxic in quails by adversely affecting their reproductive system (Hoshi *et al.*, 2014). During exposure to some neonicotinoids like imidacloprid, the variations in the embryonic development phase, hatchability letdown, genetic abnormalities, morphological and skeletal malformations have been reported. The developmental anomalies include over-sized head, twisted limbs, scanty feathers on the embryo, visceral ectopia, yolk sac failed retraction, and beak deformity (Hussein and Singh, 2016). In bobwhite quails, imidacloprid has affected embryonic development by causing anatomical deformities and altered organ masses (Gobeli *et al.*, 2017). Similarly, these structural defects and teratogenicity are also endorsed by thiacloprid neonicotinoid in chicken embryos. The vital organs including lungs and liver of infected embryos show various pathological abnormalities. The petechial hemorrhages, necrosis, and degenerative hepatic tissues can be seen in the liver of experimentally treated embryos (Salvaggio *et al.*, 2018).

As an experimental model, the chick embryo is commonly used for research purposes in biological studies because of its readily availability, cost-efficiency, and an alternate approach to the treatment of laying hen. Due to absentia of maternal metabolic activity, it requires the administration of lesser amounts of constituents for the testing of any insecticide (Petrovova *et al.*, 2016). As there is no previous documented literature available regarding the teratogenic effects of thiamethoxam in the chicken embryo, this study had been designed to evaluate the impact of TMX on embryonated eggs.

MATERIALS AND METHODS

Chemical: The thiamethoxam (TMX) was purchased from the local market with the same product name "Thiamethoxam® WG25%", manufactured by Four Seasons Chemicals, Rahim Yar Khan, Pakistan. There is no previous data available regarding the sublethal or lethal dose of TMX particularly for embryonated chicken eggs. As other members of the neonicotinoid's class comprising mainly of thiacloprid and imidacloprid were proved to be teratogenic in chicken, mice, and fish embryos. So, three relative doses were prepared accordingly (Hussein *et al.*, 2014; Salvaggio *et al.*, 2018).

Experimental plan: This study was carried out on 100 embryonated eggs (72 hours old) of the commercial broiler (ROSS). The eggs were equally divided into five groups i.e., A, B, C, D, and E. The group A and B were kept as negative control and sham control, respectively. The defective or unfertilized eggs were discarded by the candling method. The exact location of the air cell is outlined on eggshell by a led pencil. After that, the eggs were thoroughly cleaned with 70% alcohol solution. A hole was carefully drilled with a sterile thumb pin over the air cell boundary of each egg. A sterilized insulin syringe was inserted horizontally for administering purpose. Sham group was administered with normal saline 20µl/egg for validation purpose. The increasing concentrations of TMX at a dose rate of 20, 50, 100, and 150µl/egg was administered to group B, C, D and E, respectively (Table 1) was administered in air cells of the embryonated eggs (Hussein and Singh, 2016; Salvaggio *et al.*, 2018). The eggs were immediately sealed with the candle wax. After that the embryonated eggs were incubated for 20 days.

Table 1: Experimental Design

Sr. No.	Groups		Treatments
1	A	Negative Control	Untreated
2	B	Sham Control (NS)	20µl/egg
3	C	TMX	50µg/egg
4	D	TMX	100µg/egg
5	E	TMX	150µg/egg

Note: All the experimental groups contained equal number of eggs (20 eggs/group).

Post-treatment incubation: A semi-automatic indigenously made incubator having a thermostat, air circulation fan and hygrometer was used for the incubation of treated and control groups eggs. These eggs were incubated under standard conditions at an appropriate temperature (38°C±0.5°C) and 55-60% humidity was maintained with no additional O_2 or CO_2 (Hussein *et al.*, 2015). The broad ends of eggs were placed slightly upwards and tilted 2-3 times a day except for the last 3 days of incubation. The humidity was increased up to 70% in the hatching phase. The embryos were opened at 20th day of incubation to check any embryonic abnormalities or growth retardation. Any mortality which occurred during this incubation period, the dead embryos were subjected to further analysis accordingly.

Parameters evaluated: On 20th day of incubation, the embryos were thoroughly observed for body deformities and teratogenicity as per Olkowski *et al.* (2013). Physical parameters which were evaluated includes embryonic weight, crown-rump length, head size, beak length and shank length. Gross examination of liver was performed and then fixed in 10% neutral buffer formalin solution for the histopathological studies (Bancroft and Gamble, 2008).

Data Analysis: The teratological parameters were qualitatively evaluated by preparing a scorecard as per Olkowski *et al.* (2013). The collected data from the above-mentioned parameters were assessed by one-way ANOVA. Furthermore, the Tuckey's test ($p \leq 0.05$) was used to check significance and to compare group means. The SAS® University Edition software was used for this purpose (Der and Everitt, 2015).

RESULTS

The developmental and teratological parameters were evaluated on 20th day of incubation. The experimental groups treated with higher doses of TMX and in later embryonic stages showed more promising results as compared to others (Table 2). The mortality rate was increased during the late stages of embryonic development and treated with higher concentrations of thiamethoxam. No mortality was observed in the control negative group and sham group. The highest mortality was recorded in group E (40%) followed by the group D (15%) and C (10%).

The physical body parameters of experimental groups were compared with the control negative group. A significant decreasing trend in embryonic weight and crown-rump length (CRL) of all treated groups was observed as the concentration of TMX was increased (Table 3). For the evaluation of shortness of beak, it was measured from the beak tip up to the nostrils. A gradual but non-significant decrease in relative beak length was evident in all treated groups except group E (received highest dose of TMX). Only group E showed significantly shorter relative beak length as compared to the control group. Anterior to posterior head length was measured for the evaluation of head enlargement. The main concern of this study was to evaluate relative head length, which was calculated after comparing it with CR-length. The experimental groups treated with higher doses of TMX showed a comparatively enlarged head. The relative head length was significantly more in group D and E treated with TMX @ 100µg and TMX @150µg, respectively. Similarly, relative shank length was measured from the tibiotarsus joint up to the toes base for the evaluation of shortness of shank. The relative shank length of group D and E showed significant reduction against control group.

Teratological parameters: The teratological parameters were thoroughly assessed and compared with control groups. Multiple teratogenic effects were combinedly observed in some chicken embryos. On a qualitative basis, the severity of teratogenicity and its percentage occurrence was calculated by preparing a score-card. The highest teratogenic percentage was observed in group E (TMX @ 150µg). Based on the embryonic weight and CR-Length, the chick embryos were considered as growth retarded in relation to control group. The qualitative analysis of chick embryos on the 20th day showed that the severity of growth retardation gradually escalated with the increasing TMX dose rate as shown in (Fig. 1). A chick embryo in group E (TMX @ 150µg) was found with exencephaly and left eyed anophthalmia (Fig. 2). Furthermore, the beak agnathia, limb deformities, retraction of yolk sac failure, and scanty Feathers were also observed (Fig. 3).

Table 2: Physical body parameters in 20th day old chicken embryos treated with sub-lethal doses of thiamethoxam

Groups	A	B	C	D	E
Embryo Weight (g)	41.03±1.03^{a}	38.56±1.22^{ab}	36.44±1.29^{b}	34.62±1.30^{b}	27.34±5.01^{c}
CR-Length (cm)	8.89±0.40^{a}	8.34±0.28^{ab}	7.94±0.23^{bc}	7.26±0.40^{c}	5.80±0.75^{d}
Beak length (cm)	0.60±0.03^{a}	0.56±0.02^{ab}	0.52±0.02^{bc}	0.47±0.03^{c}	0.36±0.06^{d}
Head length (cm)	2.69±0.08^{a}	2.56±0.10^{ab}	2.47±0.09^{ab}	2.32±0.10^{b}	1.96±0.28^{c}
Shank length (cm)	3.11±0.12	2.86±0.14^{ab}	2.67±0.12^{b}	2.40±0.11^{c}	1.79±0.24^{d}
Relative beak length	6.69±0.07^{a}	6.67±0.16^{a}	6.59±0.09^{a}	6.49±0.17^{a}	6.22±0.33^{b}
Relative head length	30.31±0.86^{c}	30.75±0.80^{bc}	31.10±0.53^{bc}	31.98±1.21^{b}	33.66±0.76^{a}
Relative shank length	35.04±0.68^{a}	34.27±0.73^{ab}	33.67±1.00^{ab}	33.08±0.40^{bc}	30.78±1.49^{c}
Absolute liver weight (g)	0.96±0.02^{a}	0.90±0.02^{ab}	0.87±0.02^{ab}	0.84±0.03^{b}	0.68±0.13^{c}
Relative liver weight	2.33±0.01^{c}	2.34±0.02^{c}	2.38±0.06^{bc}	2.43±0.06^{ab}	2.50±0.08^{a}

Note: In each column, the values (means ± SD) with different superscript letters are statically significant (P≤ 0.05).

Table 3: Qualitatively analysis of teratogenic effects in chicken embryos

Teratogenic Effects (Scorecard)	Groups				
	(A) Control –ve	(B) Sham Group NS @ 20µL	(C) TMX @ 50µg	(D) TMX @ 100µg	(E) TMX @ 150µg
			Severity *		
Growth Retardation	-	-	++	++	+++, ++++
Head Enlargement	-	-	-	+	++
Deformed Head (Exencephaly)	-	-	-	-	++++
Eye Defects (Anophthalmia)	-	-	-	-	++++
Beak Deformities (Agnathia)	-	-	-	-	++++
Limb Deformities	-	-	-	-	++
Scanty Feathers	-	-	-	+	+++
Retraction of yolk sac failure	-	++	++	+++	+++, ++++
Teratogenicity %	0%	0%	5%	10%	30%

* + Mild, ++ Moderate, +++ Severe, ++++ Very Severe

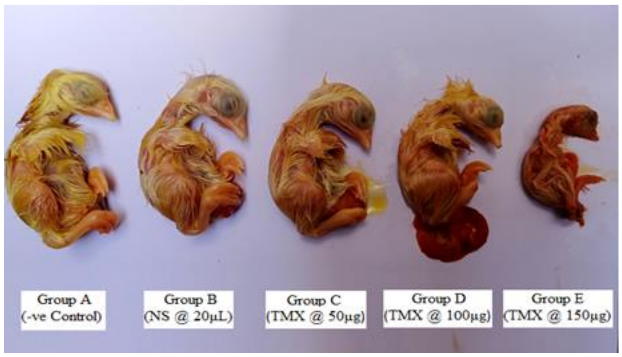

Fig. 1: Photograph of chicken embryos showing gradual reduction in body size

Fig. 2: Photograph of chicken embryo showing exencephaly, beak aganathia and anophthalmia.

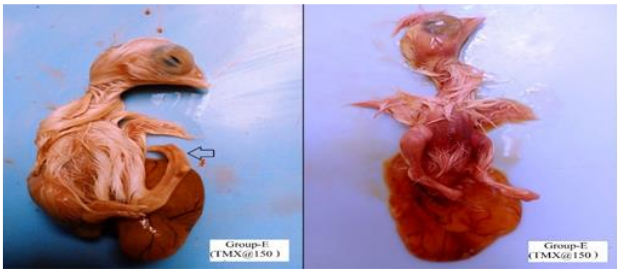

Fig. 3: Photograph of chicken embryos showing limb deformity, retraction of yolk sac failure and subcutenous hemorrhages.

Gross and histopathological changes in Liver: The relative weight of embryonic liver was calculated for the actual evaluation of liver size against embryonic weight. A significant enlargement of liver size was quite evident in group D and E (Table 2). The liver of experimentally treated groups showed petechial hemorrhages and congestion with significant enlargement in size. Histopathological changes those were found in the liver tissue include moderate to severe congestion, fatty changes (steatosis), increased sinusoidal spaces and vacuolar degeneration (Fig. 4A-4D). Necrosis was also evident with degenerated hepatocytes.

DISCUSSION

In the agriculture sector, extensive usage of neonicotinoids (neonics) has posed a serious threat to poultry birds in terms of toxicity (Cimino *et al.*, 2017). Poultry birds are generally become affected due to bioaccumulation of neonics in agricultural crops like wheat, maize, and barley etc. (Hallmann *et al.*, 2014). Although thiamethoxam, imidacloprid and clothianidin are banned by European Union and EPA (Environmental Protection Agency) in various American states, due to adverse effects on non-targeted species. In the developing countries, no authority has yet batted an eye on the unprecedented usage of neonicotinoids in the field crops or seed dressings (Naveed *et al.*, 2010; Filazi *et al.*, 2017).

Initially, the neonics were considered safest choice for pest management until their agonistic effects were discovered on non-targeted species. Neonics selectively acts on the postsynaptic receptors of nicotinic acetylcholine (nAChRs) located in the central nervous system. It causes the blockage of nAChRs by sidestepping the chemical messenger ACh (acetylcholine) and binding at the receptor site. This process leads to paralysis, and ultimately death of an insect occurs (Jeschke *et al.*, 2011). In comparison, neonicotinoids have relatively poor blood-brain barrier penetration (BBB) in vertebrates so the acute toxicity of the neonicotinoids is mainly linked to the α7 potency of the nAChRs subtype. The vertebrates have nAChRs placed in skeletal muscle, brain, spinal cord, and autonomic ganglia (Tomizawa and Casida, 2011). High dosage of neonics may cause dizziness, labored breathing, apathy, or death in mammals. Although the birds have a lesser affinity towards neonics due to variability in receptor subtype, severe toxicological effects have been reported in higher concentrations.

Now-a-days, the teratogens are being considered as a greater health concern in mammals and bird populations. Various teratological studies have been performed previously to evaluate the developmental effects of neonicotinoids using chick embryo as a research model (Davey and Tickle, 2007). Administration of suspected teratogen into embryonated eggs for the teratogenicity evaluation is considered as an alternative and better approach, rather than experimental treatment of laying birds (Petrovova *et al.*, 2016). Amongst neonicotinoids, the clothianidin, imidacloprid, and thiacloprid have been previously used for teratological studies in zebrafish, mice, quails, and chicken embryos. TMX lowered the feed consumption and impaired the liver function in laying hens. The egg production was significantly reduced, and thinness of eggshells was also observed (Adejumo and Ologhobo, 2015; Gul *et al.*, 2020). Thiamethoxam caused embryotoxicity and arrested growth in mice and rabbits (Babeľová *et al.*, 2017). There is no data available regarding the teratogenic effects of TMX in chicken embryos.

The objective behind this research was to evaluate teratogenic effects caused by thiamethoxam in chicken embryos. Imidacloprid and thiacloprid have been used previously for teratological studies. Imidacloprid showed teratogenicity at the dose rate varying from 5-50µg (Hussein *et al.*, 2014). While thiacloprid at 0.92, 9.2, 92µg caused developmental anomalies in chick embryo (Salvaggio *et al.*, 2018). So, the experimental doses were prepared accordingly. In-ovo administration of TMX was performed in chicken embryos by diluting 50µg, 100µg, and 150µg TMX in 20µL of distilled water. After that eggs were incubated at appropriate temp. (38±0.5°C) and humidity (55-70%) for 20 days as per (Hussein *et al.*, 2015).

The results at 20th day showed significant variations in physical body parameters. Gradually increased mortality was seen in chick embryos directly proportional to a higher dosage of TMX-treated groups. A decreasing trend was observed in embryonic weight and crown-rump length of experimental groups. The sham group exhibited

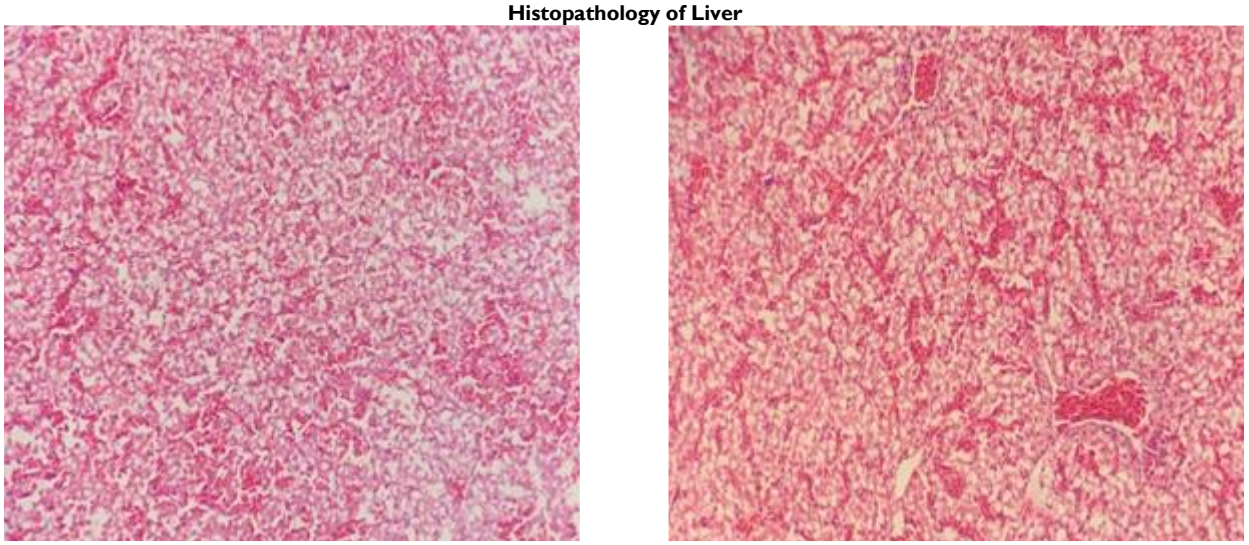

Fig. 4A: Photomicrograph of embryonic liver from group E (20x) showing severe congestion, steatosis and vacuolar degeneration.

Fig. 4B: Photomicrograph of embryonic liver from group D (20x) showing moderate congestion, mild steatosis and vacuolar degeneration.

Fig. 4C: Photomicrograph of embryonic liver from group C (20x) showing mild congestion, mild steatosis and vacuolar degeneration.

Fig. 4D: Photomicrograph of embryonic liver from group A (20x) showing normal hepatic parenchyma.

no significant alterations in embryogenesis as expected. These results are also endorsed by imidacloprid when used for teratological studies in chick embryos. Moreover, the enlarged head, shortened beak, and shank were also amongst significantly observed parameters (Hussein and Singh, 2016). Head enlargement and shorter shank length occurred due to defective osteogenesis caused by neonicotinoids as mentioned by Wang *et al.* (2016).

Qualitative analysis of teratological parameters was performed by a scorecard as per Olkowski *et al.* (2013) Teratological effects those were observed include growth retardment, deformed head, eye anomalies, limb deformities, scanty feathers, beak malformations and failed retraction of the yolk sac (Davey *et al.*, 2018). Most of the results were previously observed by imidacloprid and thiacloprid in chicken embryos of 19^{th}-20^{th} days (Salvaggio *et al.*, 2018). The chicken embryos of group D and E were clearly seen with scanty feathers due to TMX toxicity. Scanty feathers have been previously reported in all teratological studies. Normally the yolk sac is completely retraced in the embryonic body till 20^{th} day of incubation. The failed retraction of the yolk sac was observed in all treated groups. The severity was related directly to the thiamethoxam concentrations. Visceral ectopia was not found in this experiment but has been reported formerly by other neonics (Hussein *et al.*, 2014). Exencephaly was observed due to gradual deteriorative changes occurred in the cerebellum as mentioned by Singh *et al.* (2015). In this condition, the brain was exposed outside of the skull. Agnathic beak also resulted due to the toxicopathological impacts of TMX on neural crest cells in brain (Wang *et al.*, 2016). The same chick embryo which was found with exencephaly also showed a severe form of anophthalmia, in which one or both eyes were completely failed to develop. Anophthalmia is mostly observed as a birth defect in teratogens-treated chick embryos. Limb deformities including underdeveloped and twisted limbs were also supported by the thiacloprid results (Salvaggio *et al.*, 2018).

The toxic chemicals when delivered through the In-ovo route, mainly affect the liver tissue as mentioned by Kedam and Chittoor (2017). Wherefore the gross and histopathological analysis was performed only for the hepatic tissues. The relative enlargement of the liver was evident in embryos treated with a higher dosage of TMX. The obtained results were comparable to imidacloprid related study on quails (Gobeli *et al.*, 2017). The histopathological evaluation of liver showed remarkably

altered tissues as compared to control groups. The liver enlargement along with mild to severe congestion and degenerative hepatocytes was observed in experimental groups, treated with a higher dosage of TMX. Mild to severe steatosis and vacuolar degeneration was also detected in hepatic tissues with increased sinusoidal spaces. Similar changes were also reported in the liver sections of laying hens treated with thiamethoxam by Adejumo and Ologhobo (2015). Histopathological changes in the liver caused by thiamethoxam were analog to as of thiacloprid in the chicken embryo, in which petechial hemorrhages and cellular infiltration were also remarked (Salvaggio *et al.*, 2018).

Conclusions: This study has concluded about the teratogenic effects of TMX in chicken embryos in the pre-existing list of teratogenic neonicotinoids. The chicken embryos treated with a lower dosage of TMX or in early embryonic stages showed no remarkable teratogenicity other than growth retardation. Comparatively higher doses of TMX were found to be significant teratogenic causing developmental defects and histopathological alterations in liver of chick embryos. Therefore, it is recommended that excessive usage of TMX as an insecticides/pesticides in the poultry sheds keeping in view the health and welfare of the poultry flocks. In a broader aspect it will improve human health through the curtail of residual effects from contaminated eggs and meat.

Authors contribution: MAZ, STG, AK, MA and BM were actively involved in idea conceiving and project designing and execution. MZ, MI and FAA were involved in data analysis, interpretation and write up of the manuscript. All authors approved the manuscript.

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