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Down-regulation of immuno-linked genes in acutely dimethoate exposed Kamrupa chicken

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ABSTRACT

The present study evaluated the acute toxic effects of dimethoate and its modulation on genetic expression on the immune status in Kamrupa chickens. A total of 20 Kamrupa chickens were divided into two groups, each consisting ten birds: group 1 served as control while group 2 as treatment and received a single LD_{50} dose (48 mg/kg body weight) of dimethoate per os. The birds were subjected to examination of haemato-biochemical and histopathological changes as well as status of genetic expression pertaining to immunity. Birds exposed to dimethoate exhibited rapid onset of depression, anorexia, diarrhea, excessive salivation, and incoordination, ultimately leading to death. Haematological analysis showed elevated haemoglobin, total erythrocytic count, total leucocytic count, and heterophil levels, with decreased lymphocyte levels. Serum enzyme analysis indicated elevated Alanine amino transferase, Aspartate amino transferase, Alkaline phosphatase, Total cholesterol, and Uric acid levels, while Serum cholinesterase levels were decreased. Postmortem examination revealed liver, kidney, and brain congestion, haemorrhage, and hypertrophy, with liver and kidney showing degenerative changes and the brain exhibiting inflammatory lesions. Gene expression studies indicated downregulation of immune-linked *Avian Beta Defensin 1*, *Avian Beta Defensin 6*, and *Avian Beta Defensin 7* genes. The results of the present study suggest that acutely dimethoate exposed Kamrupa chicken had profound harmful effects with down regulation of immune-linked genes.

Keywords: Acute toxicity, Dimethoate, Gene expression, Haemato-biochemical, Histopathology, Immune status

Pesticides, known to be indispensable player in modern agricultural practices, have become fundamental instrument for crop safeguarding and disease control on a global scale. Their multifaceted compositions and wide-ranging uses are crucial in enhancing agricultural output by controlling pests that not only threaten crop yield, but also serve as carriers for illnesses harmful to human and animal. Nevertheless, the widespread application of pesticides, although beneficial for increasing food production and controlling the spread of diseases, gives rise to significant apprehensions regarding their considerable impact on the environment and human health (Adam *et al.* 2024).

Dimethoate, also known as O, O-dimethyl-S-N-methylcarbamoylmethyl-phosphorodithioate, is an organo-phosphorus insecticide that acts both as contact and systemic poison is a commonly available and widely used OP compound in Indian agriculture. It is extensively employed to combat a wide variety of insects and mites and is also utilized for the indoor management of houseflies. The primary at-risk populations for increased exposure to larger doses of dimethoate are individuals involved in its production, pesticide workers, and farm proprietors (Mancini *et al*. 2022). Although widely utilized in agriculture and domestic settings, there is currently a lack of research on its impact on human health, specifically about oxidative stress. Pesticides are crucial for maintaining food security, the excessive use of pesticides in regions like Assam poses a risk of environmental contamination and endangers the welfare of animals and birds that depend on untreated or inadequately treated pesticides (Hassaan *et al.* 2020).

Many studies have shown immune-suppression in organophosphate toxicity in avian species. Kamrupa bird is a native variety of chicken found in Assam. Therefore, this study intended to examine the effect of dimethoate on haemato-biochemical alterations as well as histological and molecular alterations to ascertain genes responsible for immune-suppression in this avian species. The findings will provide insights into the effects of such pesticides on birds and an alarm to monitor agricultural practices which can ultimately safeguard public health.

MATERIALS AND METHODS

Chemicals: Dimethoate (Roger 30% EC, based on

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85% w/w, a.i) procured from Cheminova Private Limited, Punjab, India

Experimental animals: The study was approved by the Institutional Animal Ethics Committee (IAEC) vide approval no. 770/GO/Re/S/03/CPCSEA/FVSc/AAU/IACE/22-23/1043 dated 23/03/2023, College of Veterinary Sciences, AAU, Khanapara, Guwahati. A total of 20, day-old Kamrupa chickens of either sex were included in the experiment. The birds were acclimatized for 7 days prior to experimentation in our institutional farm. Balanced nutritional diet and water were given *ad lib*. during this period. Birds were vaccinated with Ranikhet disease vaccine on 7th day.

Experimental design: Prior to experimentation, LD_{50} was determined as per OECD 425 guidelines. On 8th day, the birds were divided into two groups each consisting of 10 birds. Group I served as control and group II as treated and fed with single dose of dimethoate at the rate of 48 mg/kg body weight (LD_{50}).

Sample collection and assessment of haematological and enzyme profile: Blood samples were collected from jugular veins of all the treated groups at 0, 3, 6, 12, 24 and 36 h after dosing. Automated Hematology Cell counter (Model MS4S) was used for the analysis of Haemoglobin (Hb), Total erythrocyte Count (TEC), Total Leukocyte Count (TLC), Lymphocyte and Heterophil level. Serum activities of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), Total serum cholesterol (TC), Uric acid and Acetylcholinesterase (AchE) were analyzed using commercially available biochemical kits (Kee Diagnostics) with the help of Double Beam UV-VIS spectrophotometer (Systronics).

Post-mortem and histopathology: For gross examination, all the birds including those that died during the period of the experiment, and those that survived till the end of the experiments were sacrificed and gross alteration if any was recorded through postmortem examinations. For histopathological examination, following post-mortem, representative pieces of liver, kidney, and brain were collected in clean sample containers containing 10% formalin with proper precaution without damaging the tissues and processed by existing method (Luna, 1968).

Gene expression studies using qRT-PCR: Total RNA was extracted following the manufacturer's protocol using TRI reagent from the RNA later-preserved samples. Complementary DNA (cDNA) was synthesized as per existing protocol (Darwish *et al.* 2010) followed by Real-Time PCR. The formula outlined by Livak and Schmittgen (2001) was used to compute alterations in the expression levels of genes, and the results were presented as fold change. Primer sequence for specific genes are presented in Table 1.

Statistical analysis: Data were presented as Mean±SE. Statistical analysis of all the grouped data was evaluated using One-way analysis of variance (ANOVA) with SPSS V 26.0 software. A value of $p \leq 0.05$ was taken as statistically significant.

RESULTS AND DISCUSSION

Clinical signs: Within 2 h of the single dimethoate dosing, the chickens started showing toxicity signs such as initial excitation followed by depression, drooping of wings, incoordinated movement, and frothy salivation, sitting on their hocks with their toes curled before dying from convulsions, tremor, incoordination and recumbency. 50% of the birds died within 36 h. During short-term exposure, the primary mechanism of organophosphate (OP) toxicity is the permanent suppression of acetylcholinesterase (AChE) activity. This inhibition leads to build up of acetylcholine (ACh) and the onset of immediate muscarinic and nicotinic effects such as muscle weakness, excessive salivation, diarrhea, tremor, convulsion, and recumbency. Similar findings were reported earlier in rats. The primary mechanism of toxicity in sub-chronic or chronic exposure to OP had been linked to production of oxidative stress (Kachave *et al.* 2022).

Dimethoate-induced hematological alterations: Haemoglobin level increased in dimethoate-exposed group as compared to the control from 12 h onward. TEC also increased in the dimethoate group from 12 h onward and the increase was time dependent till 24 h. Similarly, time-dependent increase in TLC was observed in the dimethoate group from 24 h onwards. In addition, time-dependent increase in per cent of heterophil was observed in the dimethoate group from 24 h onward. On the contrary, lymphocytes count decreased significantly and time-dependently after 12 h onward (Table 2, $p<0.05$).

The increase in haemoglobin, TEC and TLC observed in the present study could be due to dehydration caused by diarrhea and excessive salivation which ultimately led to hemo-concentration. An increase in haemoglobin concentration was reported earlier in mice fed with single dose of another OP compound quinalphos (Rao and Madhavleetha 2017). However, our findings are not in agreement with report on layer chicken fed with chlorpyrifos @8 mg/kg BW for 4 weeks where haemoglobin and TLC

Table 1. qRT-PCR primer sequence of genes of interest

Primer	Accession number	Primer sequence		Product size
		Forward	Reverse	
Avian Beta Defensin 1 (*AvBD1*)	NM_204993.1	CCTGTGAAAACCCGGGACA	GCACAGAAGCCACTCTTTCG	145
Avian Beta Defensin 6 (*AvBD6*)	NM_001001193.1	TTGCAGGTCAGCCCTACTTT	CCGGTAATATGGCCACCGAC	95
Avian Beta Defensin 7 (*AvBD7*)	NM_001001194.1	ATTTCACATCCCAGCCGTGG	AGGCCTAGGAATGAAGGGCT	103
ACTB (housekeeping)	L08165	CCCATCTATGAAGGCTACGC	TCCTTGATGTCACGCACAAT	152

Table 2. Haematological alterations (Mean±SE) in control vs dimethoate exposed chickens

Time of blood collection (h)	Haemoglobin (g%) Control	Haemoglobin (g%) Acute Dimethoate Group	TEC (10^6/µL) Control	TEC (10^6/µL) Acute Dimethoate Group	TLC (10^3/µL) Control	TLC (10^3/µL) Acute Dimethoate Group	Heterophil (%) Control	Heterophil (%) Acute Dimethoate Group	Lymphocyte (%) Control	Lymphocyte (%) Acute Dimethoate Group
0	9.56±0.11	9.91±0.05c	1.49±0.22	1.81±0.05c	26.53±0.18	26.29±0.20c	4.71±0.12	4.67±0.06d	86.30±0.13	86.66±0.17ab
3	9.88±0.11	9.69±0.13bc	1.95±0.17	2.11±0.15c	26.03±0.26	26.65±0.30c	4.32±0.07	4.69±0.16d	86.72±0.22	86.80±0.13a
6	10.12±0.24	10.51±0.16bc	2.14±0.31	3.32±0.38b	25.61±0.26	27.11±0.95c	4.59±0.13	4.53±0.13d	86.28±0.40	84.00±0.91bc
12	10.72±0.13	11.32±0.12*bc	2.79±0.20	3.33±0.11*b	26.47±0.25	27.29±0.42c	4.19±0.12	5.78±0.42c	87.01±0.73	84.25±0.25*abc
24	11.24±0.28	12.34±0.15*ab	2.63±0.13	4.13±0.29*b	27.10±0.26	32.96±0.32*b	3.53±0.01	8.61±0.15*b	86.10±0.39	83.75±0.48*c
36	10.96±0.34	13.25±0.19*a	2.67±0.18	5.32±0.09*a	27.52±0.05	35.25±0.32*a	4.40±0.18	13.53±0.10*a	86.15±0.28	77.33±0.99*d

level were found to be decreased (Begum *et al*. 2015). The time-dependent decrease in the level of lymphocyte and conversely, the increase in the level of heterophil in the current study could be due to stress caused by degenerative injury leading to suppression of the immune system. Similar findings were also observed by other workers during experimental feeding of chlorpyrifos to white leghorn birds (Lee *et al.* 2013).

Dimethoate-induced biochemical alterations: There was a time-dependent increase in the level of ALT in the dimethoate group as compared to control from 6 h onward. AST level also increased time-dependently from 12 h onwards while, Serum Alkaline Phosphatase level increased from 6 h onwards in the dimethoate group. On the other hand, cholinesterase level decreased time-dependently from 12 h onward. The time-dependent increase in total cholesterol level was from 6 h onwards and that of uric acid level was from 12 h onward in the dimethoate-group (Table 3, $p<0.05$).

Significant elevation of biomarkers of liver such as aminotransferases (ALT and AST) and alkaline phosphatase by dimethoate indicates that injury to hepatocytes led to discharge of aminotransferases from their cell membrane into the bloodstream, since, an elevated level of such enzyme was reported to signal potential liver damage (Manal *et al*. 2008). Similar findings were earlier reported in rats during an experimental dimethoate toxicity study (Ahmed and Nasr 2009). Another possible explanation of the elevated enzyme levels might be due to damage of free radical scavenging system by dimethoate leading to oxidative stress as reported earlier in birds exposed to chlorpyrifos (Bharati *et al*. 2011).

The significant elevation of serum uric acid in dimethoate-exposed birds indicate that kidney function was impaired in the birds leading to inefficient filtration and thereby, accumulation of uric acid in the blood. Similar findings were reported earlier with chlorpyrifos and diuron in chicken (Nahhal and Lubbad 2018).

Normally, high density lipoprotein (HDL) transports cholesterol from the cells to the liver, where it undergoes breakdown and is eliminated from the body as a waste product. This transport system is impaired when the liver is damaged leading to accumulation of cholesterol in the blood as was observed in the present study with dimethoate. A very high cholesterol level was earlier reported in broiler birds fed with profenofos (Kafle *et al.* 2017). As with other OP compounds, dimethoate also inhibits cholinesterase enzyme and therefore, a reduction in the level of cholinesterase level in the present study is in agreement with an earlier study (Smith *et al.* 2018).

Dimethoate-induced histopathological alterations: Histopathological examination of liver showed perivascular lymphoid aggregation of hepatic parenchyma (Fig. 1), focal aggregation of mononuclear phagocytic cells, dilation of sinusoids with presence of pyknotic nuclei (Supplementary Fig. 1). In the kidneys, there was formation

Table 3. Biochemical alterations (Mean±SE) in control vs dimethoate exposed chickens

Time of blood collection (h)	ALT Control (IU/L)	ALT Acute Dimethoate Group (IU/L)	AST Control (IU/L)	AST Acute Dimethoate Group (IU/L)	ALP Control (IU/L)	ALP Acute Dimethoate Group (IU/L)	CHE Control (IU/L)	CHE Acute Dimethoate Group (IU/L)	Total Cholesterol Control (IU/L)	Total Cholesterol Acute Dimethoate Group (IU/L)	Uric Acid Control (mg/dl)	Uric Acid Acute Dimethoate Group (mg/dl)
0	24.83±0.15	25.61±0.19e	50.63±0.20	50.49±0.26d	106.08±0.38	105.83±0.07e	441.68±0.56	442.36±0.37ab	142.80±0.23	141.53±0.57e	7.91±0.14	8.32±0.14d
3	24.98±0.25	25.37±0.24e	51.34±0.28	52.34±0.39d	105.50±0.29	108.06±0.71de	440.82±0.35	444.18±0.98a	143.37±0.66	142.11±0.93e	7.70±0.28	8.12±0.21d
6	25.64±0.40	30.75±0.75*d	51.50±0.50	52.65±0.36d	106.28±0.27	111.30±0.98*d	440.03±0.51	441.06±0.82b	145.56±0.64	151.56±1.53*d	8.36±0.10	11.86±1.06c
12	25.37±0.25	48.58±0.23*c	52.56±0.37	58.50±0.50*c	107.30±0.72	160.25±0.77*c	441.10±0.17	411.86±0.64*c	146.58±0.79	161.08±1.78*c	7.52±0.11	12.07±1.05*c
24	27.13±0.30	59.75±0.29*b	52.39±0.25	97.46±0.47*b	107.10±0.57	242.09±0.965*b	439.81±0.78	391.23±0.62*d	146.64±0.41	181.54±0.46*b	7.33±0.19	18.79±0.32*b
36	26.91±0.54	77.73±0.22*a	51.97±0.56	123.87±1.17*a	107.21±0.28	293.19±0.92*a	439.12±1.16	342.89±0.56*e	148.96±0.42	196.98±1.12*a	7.59±0.15	26.35±0.48*a

Fig. 1. Perivascular lymphoid aggregation of hepatic parenchyma with focal aggregation of mononuclear phagocytic cells and presence of pyknotic nuclei (Hematoxylin and Eosin 40×) in the liver of dimethoate exposed birds.

of the cystic cavity in the glomeruli with hemorrhage of the renal parenchyma characterized by the presence of free RBC in the interstitial tissue (Fig. 2). Extensive hemorrhage and perivascular aggregation of mononuclear phagocytic cells were prominent (Supplementary Fig. 2). The histopathological changes in the brain depicted mild to moderate degeneration with necrosis of purkinjee cells (Fig. 3). Encephalitis characterized by infiltration of lymphocytes was prominent (Supplementary Fig. 3). Presence of pyknotic nucleus in the liver on histopathology indicates necorsis of hepatocytes induced by dimethoate resulting shrinkage of chromatin and nucleus. Besides, perivascular aggreagation of mononuclear phagocytic

Fig. 2. Formation of the cystic cavity in the glomeruli with atrophy of Glomerular turf, haemorrhage of the renal parenchyma and perivascular aggregation of mononuclear phagocytic cells (Hematoxylin and Eosin 40×) in the kidney.

Fig. 3. Encephalitis with lymphocytic infiltration in the brain (Hematoxylin and Eosin 40×).

cells in the kindey, encephalitis in the brain tissue with lymphocyte infiltration were some of the important findings that corroborate earlier observations (Zapadia *et al*. 2014). Brain histopathology depicted mild congestion, hemorrhage, neuronal degeneration and vacuolation. Similar alterations were reported in reproductive toxicity study of endosulfan in male albino rats (Choudhuary *et al*. 2003).

Dimethoate induced altered gene expression: Dimethoate significantly downregulates *AvBD1*, a crucial gene in avian immunity that protects tissues from infections through immune modulation and antimicrobial action (Fig. 4A). The decreased expression of *AvBD1* in dimethoate-exposed birds indicates its impact on the immune system, contributing to toxicity.

Expression of another immune function-related gene, *AvBD6*, linked to oxidative stress and inflammation, was notably downregulated in this study (Fig. 4B). Similarly, *AvBD7*, responsible for antimicrobial and immunomodulatory activities, also showed profound downregulation, as depicted by the fold change in gene expression (Fig. 4C).

Avian Beta Defensin 1 (*AvBD1*) is crucial for bird immunity, characterized by a compact structure stabilized by disulfide bonds that confer strong antibacterial activity by disrupting pathogen membranes. It supports immune cell responses and mucosal homeostasis, regulated by various stimuli to protect avian tissues from infections (Johnson *et al.* 2019). Dimethoate exposure significantly reduces *AvBD1* gene expression, indicating immune system toxicity. Similarly, Avian Beta Defensin 6 (*AvBD6*) possesses broad antibacterial capabilities, with its expression significantly decreased by dimethoate, disrupting immune pathways. *AvBD7* also shows reduced gene expression with dimethoate exposure, consistent with findings in DDT-exposed chickens (Thomson *et al*. 2018).

The results of the present study suggest that acute exposure of dimethoate to Kamrupa chicken produced symptoms of toxicity with changes in haematological parameters. Serum enzymes were elevated with the exception of acetylcholine esterase which was lowered and was indicative of organophosphate compound toxicity. Dimethoate also caused down regulation of expression of *Avian β defensin 1*, *Avain β defensin 6* and *Avian β defensin 7* genes which are linked to immunological status.

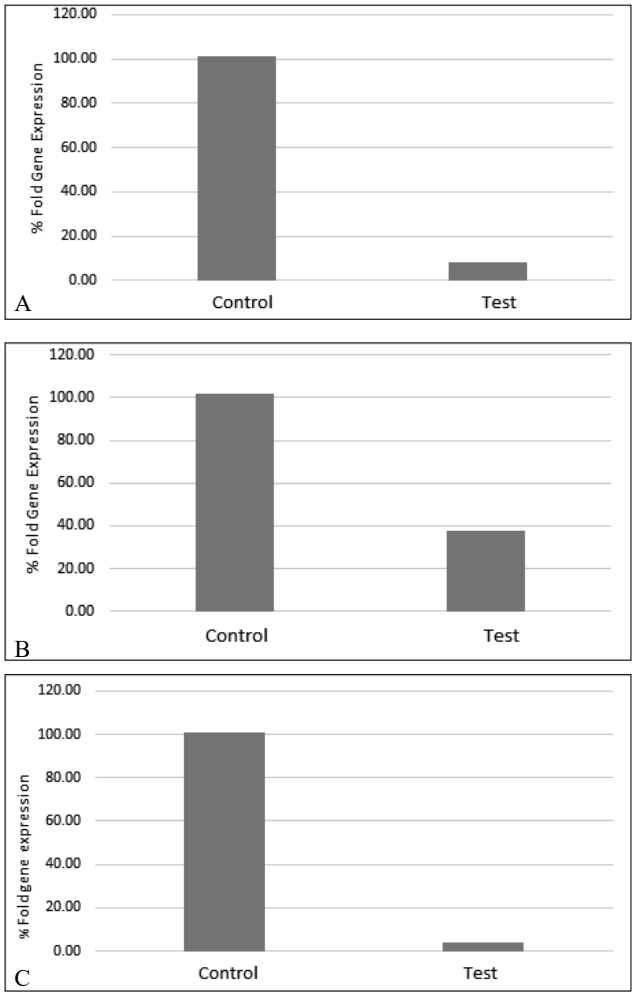

Fig. 4. Fold change (%) in gene expression of: (A) AvBD1, (B) AvBD6 and (C) AvBD7.

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