



Effect of Vitamin C Supplementation in Experimental Ionophore Toxicosis in Broiler Chickens

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ABSTRACT

Background: Ionophore toxicity is a significant but under recognized problem under field conditions and it causes significant losses to poultry farmers in terms of reduced production, immunosuppression and deterioration in quality of meat. An investigation was carried out to determine the impact of administration of toxic doses of ionophores on immunological parameters, oxidative stress response in blood and morphology of heart, skeletal muscle as well as gizzard in broilers and its amelioration by vitamin C.

Methods: For this study, 48 broiler birds, one week of age, were equally but randomly allocated to six groups. Birds in group I served as control, vitamin C @ 200 mg/litre in drinking water was administered to birds in group II, maduramicin @ 8 ppm and salinomycin @ 120 ppm were added in feed of group III and V birds, respectively. Vitamin C @ 200 mg/litre in drinking water was given in addition to maduramicin and salinomycin in groups IV and VI, respectively. The duration of the trial was two weeks.

Result: After two weeks of treatment, there was significant decrease in total protein and albumin levels along with humoral as well as cell mediated immune response in chickens fed ionophores. Also, there was significant increase in the level of antioxidant enzymes like catalase, superoxide dismutase, glutathione peroxidase and lipid peroxidation in blood, liver and kidneys in groups given ionophores alone. In addition, severe hemorrhages, necrosis and inflammation were seen in myocardium, skeletal muscles and tunica muscularis of gizzard. Vitamin C supplementation significantly reduced pathomorphological alterations, oxidative stress and improved immune response in birds intoxicated with maduramicin and salinomycin. Ionophores are often added thoughtlessly in commercial broiler ration to boost growth, but their toxicity significantly hampers broiler production. Vitamin C supplementation can be used as ameliorate agent against maduramicin and salinomycin-induced toxicity in broiler birds.

Key words: Ionophore, Maduramicin, Oxidative stress, Salinomycin, Vitamin C.

INTRODUCTION

Avian coccidiosis caused by *Eimeria* spp. is an important parasitic disease mainly affecting young birds in the age group of 3 to 6 weeks causing high morbidity and mortality (Sood *et al.*, 2009; Fatoba and Adeleke, 2018). To keep the disease under check, anticoccidial drugs mainly ionophores are added to the poultry feed as growth promoters and anticoccidiostats (Ekinici *et al.*, 2023). Maduramicin and salinomycin are commonly used ionophores which form zwitterionic complexes with polar cations such as Na⁺, K⁺ and Ca²⁺ and thus promote ionic transport across the cell membranes. The accumulation of these cations inside the parasitic cell against their concentration gradient ultimately causes death of the parasite (Gupta, 2012). The recommended dose of maduramicin and salinomycin in broiler is 5 and 60 ppm, respectively. Due to narrow safety margin, callousness in their administration as feed additives however; it may cause toxicity and promote antimicrobial resistance with public health implications (Ensley, 2020; Wong, 2019). Recently, resistance against salinomycin and maduraycin has been reported in field isolates of *Eimeria* species (Khurshed *et al.*, 2023). Ionophore toxicity diminishes appetite, weight gain and causes depression, anaemia, lymphopenia, ataxia, recumbency and death (Sharma *et al.*, 2021; 2022). Moreover, anticoccidial drugs have been shown to cause

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oxidative stress (Hajimohammadi *et al.*, 2015). Vitamin C is an easily available potent antioxidant which can alleviate oxidative stress in a variety of animal species (Wu *et al.*,

2022). Even though, indiscriminate application of ionophores in field is rampant, toxicity associated with its overuse is an under recognised problem in broilers. As such literature on adverse impact of improper administration of ionophores to chickens *via* feed is limited and there are very few reports available on the impact of ionophore toxicity on humoral and cell mediated immune response. Also, many previous studies have shown the protective response of vitamin C supplementation on overall health, growth and productivity of broilers (Srinontong *et al.*, 2024). Bearing in mind the overmedication with coccidiostat ionophores in poultry being widely practiced nowadays, this work was designed to investigate toxic effects of maduramicin and salinomycin on the health of broilers.

MATERIALS AND METHODS

Approval from animal ethics committee

The present study was conducted at Division of Veterinary Pathology, FVSC and AH, SKUAST-Jammu in the year 2022. This whole experiment was approved by Animal Ethics committee of SKUAST- Jammu, vide order no. I/AEC/2020, Dated 22-10-2020.

Experimental design

A total of 48, day old broiler chicks of either sex were bought from a commercial hatchery in Jammu. The chicks were fed *ad libitum* on a standard ration without the addition of anticoccidial ionophores. Doses of ionophores used in the current study were chosen on the basis of previously published data (Sivakumar *et al.*, 2007; Kamashi *et al.*, 2004). Clean tap drinking water was provided to birds *ad libitum* throughout the experiment. After one week of acclimatization, 48 birds were randomly divided into 6 groups *i.e.*, 8 birds in each group. Group I served as control, group II was given vitamin C @ 200 mg/liter in drinking water, group III was given maduramicin @8 ppm in feed, group IV was given maduramicin @ 8 ppm in feed and vitamin C @ 200 mg/liter in drinking water, group V birds were treated with salinomycin @120 ppm in feed and group VI were treated with salinomycin @ 120 ppm in feed along with vitamin C @ 200 mg/liter of water. Birds were further kept for a two-week period and were constantly monitored.

Blood collection

Blood samples were collected from all the birds at 2nd week post exposure (WPE) from heart. Blood was collected in sterilized test tubes with and without addition of anticoagulant for obtaining plasma and serum, respectively. Serum was collected for biochemical and immunological studies. Plasma was separated to obtain rbc's for preparation of lysate for estimation of oxidative stress markers in blood.

Biochemical studies

The serum samples were analyzed for total serum protein (Biuret method) and albumin (BCG dye binding method), alanine amino transaminase (ALT) and aspartate amino

transferase (AST), creatinine and alkaline phosphatase (ALP) using standard kits obtained from Erba Mannheim Transasia Bio-Chemicals Ltd, (H.P), India. Globulin values and albumin: globulin ratio (A: G ratio) were also determined.

Oxidative stress

Oxidative stress parameters were measured in blood, liver as well as in kidneys. Superoxide dismutase (SOD) (U/mg of Hb or U/mg of tissue) was determined as per the prescribed method by Marklund and Marklund (1974), Catalase (CAT) (mmole H₂O₂ utilized/min/mg of Hb or mmole H₂O₂ utilized/min/mg of tissue), Glutathione peroxidase (GPx) (U/mg of Hb or U/mg of tissue) and malondialdehyde (MDA) (nM MDA produced/mg of Hb/h or nM MDA produced/mg of tissue/h) were determined as per the methods of Aebi (1983), Hafeman *et al.*, (1974) and Shafiq-Ur-Rehman (1984), respectively.

Immunological parameters

Humoral immunity (HI) and cell mediated immune response (CMI) was determined by measuring haemagglutination inhibition (HI) antibody titer against the New Castle Disease Virus (NCDV) by utilizing 4HA (hemagglutination) units of Lasota strain (Allan and Gough 1974). CMI was done as per the method described by Corrier *et al.*, (1990).

Pathological studies

Gross lesions were recorded after conducting a thorough post mortem examination. Representative tissue pieces of liver, kidneys, heart, spleen, gizzard and skeletal muscle were collected in 10% neutral buffered formalin, sectioned using standard histopathology technique. Lesions in sections stained with haematoxylin and eosin (H and E) were scored quantitatively (on a scale 0-3) (Shackelford *et al.*, 2002).

Statistical analysis

Statistical analysis was done by a one-way ANOVA employing Duncan descriptive statistical analysis (Snedecor and Cochran 1994).

RESULTS AND DISCUSSION

Biochemical parameters

The alterations in total serum protein, albumin, globulin and A:G ratio in different groups are presented in Table 1. There was a significant reduction in total protein as well as albumin while globulin levels remained unaffected levels in groups fed ionophores. Vitamin C significantly improved total protein in both ionophore groups but albumin levels were significantly better only in salinomycin group. Others have also reported that concentration of total protein, albumin and globulin were decreased because of salinomycin toxicity (Arun *et al.*, 2003). The data on alterations in biochemical parameters indicative of liver and kidney damage is presented in Table 2. Creatinine, ALP, AST and ALT, levels were found to be significantly raised in birds fed maduramicin and salinomycin at 2nd WPE. Vitamin C addition significantly reduced levels of all variables except creatinine.

Oxidative stress parameters

The average data of SOD, CAT, GPx and MDA in blood of birds of different groups is summarized in Table 3. The average data of SOD, CAT, GPx and LPO in the liver of birds in different groups as observed at 2nd WPE is summarized in Table 4. There was a significant increase in values of SOD, CAT and LPO in groups fed maduramycin and sainomycin whereas significant improvement was seen with concomitant vitamin C feeding in CAT and SOD values. No significant changes were registered in GPx levels after maduramycin toxicity when compared to control birds however salinomycin caused a significant increase in GPx as compared to control. Vitamin C completely ameliorated raised GPx concentrations in salinomycin fed birds. Average SOD, CAT, GPx and LPO levels in the kidneys of birds at 2nd WPE is summarized in Table 5. Group III and V had

significantly increased SOD, CAT, GPx and LPO levels as compared to control group but vitamin C caused significant amelioration of SOD, CAT, GPx and LPO levels. Free radical mediated cell damage is an important player in the pathogenesis of various disease conditions (Sood *et al.*, 2019). Oxidative damage is kept in check by antioxidant machinery which consists of enzymes like SOD, CAT and GPx which scavenge reactive oxygen species (ROS) such as O₂⁻, H₂O₂ etc generated in response to xenobiotic metabolism (Yang *et al.* 2024). There was a significant increase in SOD, CAT, GPx and LPO values after giving maduramycin. Similar alterations were recorded in salinomycin group except for CAT levels which remained unaffected. Vitamin C supplementation partially but significantly ameliorated both the ionophore induced alterations. However, in a study by Ni *et al.* (2019),

Table 1: Effect of administration of ionophores and vitamin C on total serum protein, albumin, globulin and albumin: Globulin (A:G) ratio in different groups.

Treatment group	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A:G
Group I	3.16 ^d ±0.09	1.71 ^c ±0.06	1.45 ^{bc} ±0.97	1.03 ^a ±0.01
Group II	3.51 ^e ±0.08	1.83 ^c ±0.06	1.69 ^{cd} ±0.08	1.08 ^{ab} ±0.06
Group III	2.32 ^b ±0.07	0.98 ^a ±0.11	1.31 ^{abc} ±0.18	0.82 ^{ab} ±0.12
Group IV	2.80 ^c ±0.01	1.01 ^a ±0.17	1.79 ^d ±0.16	0.67 ^a ±0.15
Group V	2.05 ^a ±0.08	0.93 ^a ±0.03	1.12 ^{ab} ±0.10	0.88 ^{ab} ±0.19
Group VI	2.41 ^b ±0.06	1.24 ^b ±0.09	1.17 ^{ab} ±0.01	1.05 ^{ab} ±0.25

Values are given as mean ± SE of 8 birds.

Values having different superscripts (a,b,c,...) in a column are statistically different from one another at 5% level of significance.

Table 2: Biochemical parameters of birds in different groups at 2nd WPE.

Treatment group	Creatinine (mg/dl)	ALP (U/L)	AST (U/L)	ALT (U/L)
Group I	0.70 ^a ±0.01	147.48 ^a ±3.97	60.82 ^a ±2.10	11.33 ^a ±0.76
Group II	0.78 ^a ±0.04	147.55 ^a ±3.39	61.21 ^a ±2.09	10.95 ^a ±0.65
Group III	0.97 ^b ±0.03	337.03 ^c ±36.27	107.85 ^c ±5.53	24.84 ^c ±1.49
Group IV	0.96 ^b ±0.03	250.05 ^b ±20.06	91.25 ^b ±2.43	20.07 ^b ±0.29
Group V	0.90 ^b ±0.03	404.33 ^d ±23.63	137.76 ^d ±5.52	31.53 ^d ±0.83
Group VI	0.89 ^b ±0.04	216.00 ^b ±8.41	92.49 ^b ±2.63	21.64 ^b ±0.35

Values are given as mean ± SE of 8 animals unless otherwise stated.

Values having different superscripts (a,b,c,...) in a column are statistically different from one another at 5% level of significance.

Table 3: Effect of ionophores and vitamin C on blood SOD, CAT, GPx and LPO values in different groups.

Treatment group	Antioxidant biomarkers			
	SOD	CAT	GPX	MDA
	U/mg of Hb	mmole H ₂ O ₂ utilized/min/mg of Hb	U/mg of Hb	nM MDA produced/mg of Hb/h
Group I	14.46 ^a ±1.15	44.67 ^{ab} ±13.32	8.32 ^b ±0.17	4.33 ^a ±0.34
Group II	12.86 ^a ±0.81	58.10 ^{bc} ±2.46	7.85 ^b ±0.30	3.55 ^a ±0.65
Group III	51.02 ^c ±4.90	98.30 ^d ±4.53	12.35 ^c ±0.65	10.95 ^{bc} ±0.31
Group IV	25.88 ^b ±2.23	66.53 ^c ±6.06	3.94 ^a ±0.27	8.34 ^b ±0.62
Group V	32.49 ^b ±2.69	41.27 ^{ab} ±3.17	11.24 ^c ±0.31	13.29 ^c ±2.11
Group VI	9.47 ^a ±1.22	46.32 ^a ±2.31	7.94 ^b ±0.55	4.41 ^a ±0.37

Values are given as mean ± SE of 8 birds.

Values having different superscripts (a, b, c,.....) in a column are statistically different from one another at 5% level of significance.

significantly increased activities of SOD, CAT, GPx and GST in the liver of zebrafish were observed after maduramicin toxicity and Kamashi *et al.* (2004) also reported increased levels of GPx, GSH and CAT in blood in chicks receiving toxic doses of salinomycin, which are in concurrence with our results. It has been shown that addition of ginseng, can ameliorate the oxidative stress caused by maduramicin (Sivakumar *et al.*, 2007) which is similar to our results where vitamin C significantly reduced oxidative stress caused by maduramicin and salinomycin. Many previous studies have also shown that dietary supplementation with antioxidants mitigates oxidative stress and improves broiler production (Gopi *et al.*, 2019; Choudhary and Singh, 2017).

Immune response parameters

The HI test showed robust titer specific to NDV ranging from 26 to 27 in all the vaccinated groups. There was a decrease in HI titer in group III and V (26) as compared to group I and group II (27) whereas group IV and VI showed same titer as that of control group birds. The data of skin thickness measurements in various groups is presented in Table 6 and Fig 1a and 1b. A significant reduction in skin thickness in response to PHA-P inoculation in the interdigital space was seen in ionophore only administered groups. Vitamin C co-administration with either ionophore improved the humoral immune response. These results were also supported by severe lymphocyte depletion in

spleen and bursa after ionophore administration and lack of such changes in birds co-treated with vitamin C. Similar findings have also been reported by Koutoulis *et al.* (2013) who saw splenic atrophy in broiler breeders and turkeys because of salinomycin toxicity. Rahman and Joshi (2010) also showed that lead acetate suppressed both humoral response and cell mediated response in broiler birds.

Pathomorphological observations

A comparison of the lesion scores (heart, skeletal muscles, gizzard, kidney, liver and spleen) in different groups is presented in Table 7.

Heart

In group III and V, severe hemorrhages on epicardium were observed and multifocally, subepicardium had aggregates of heterophils and lymphocytes and myocardial interstitium was expanded by congestion, hemorrhages and perivascular edema. Myocardial fibres showed multifocal extensive segmental degeneration and coagulation necrosis. Cardiac myofibers had homogeneously hyalinized sarcoplasm with pyknotic or faded nuclei which were often fragmented with loss of cross striations and infiltrated by predominantly heterophils and fewer macrophages (Fig 2a, 2c). In groups IV and VI, the pathological changes were subdued as only mild congestion and degeneration was seen and focal necrotic

Table 4: Oxidative stress parameters in the liver of birds in different groups at 2nd WPE.

Treatment group	Antioxidant biomarkers			
	SOD U/mg of tissue	CAT mmole H ₂ O ₂ utilized/min/mg of tissue	GPx U/mg of tissue	MDA nM MDA produced/ mg of tissue/h
Group I	57.59 ^a ±9.21	2180.79 ^a ±122.51	152.91 ^b ±1.98	96.76 ^a ±13.52
Group II	62.89 ^a ±3.84	2078.46 ^a ±198.95	144.91 ^b ±6.98	113.28 ^a ±10.33
Group III	491.39 ^c ±80.44	2917.17 ^b ±222.78	179.73 ^b ±3.04	366.96 ^b ±78.82
Group IV	67.90 ^a ±6.97	2241.61 ^a ±103.39	140.68 ^b ±14.91	102.09 ^a ±8.42
Group V	442.57 ^c ±63.25	3976.41 ^c ±196.72	252.49 ^c ±4.42	135.58 ^c ±10.67
Group VI	200.70 ^b ±22.52	2582.74 ^b ±167.66	147.43 ^b ±9.01	93.39 ^a ±7.63

Values are given as mean ± SE of 8 animals. Values having different superscripts (a, b, c....) in a column are statistically different from one another at 5% level of significance.

Table 5: Oxidative stress parameters in the kidneys of birds in different groups at 2nd WPE.

Treatment group	Antioxidant biomarkers			
	SOD U/mg of tissue	CAT mmole H ₂ O ₂ utilized/ min/mg of tissue	GPx U/mg of tissue	MDA nM MDA produced/ mg of tissue/h
Group I	311.57 ^a ±18.13	1948.32 ^a ±54.29	187.37 ^b ±3.09	78.266 ^a ±5.95
Group II	299.33 ^{ab} ±23.15	1863.16 ^a ±164.03	196.75 ^b ±8.91	85.71 ^a ±11.91
Group III	847.74 ^d ±21.23	3974.73 ^b ±282.11	233.26 ^c ±5.60	389.95 ^d ±50.25
Group IV	273.71 ^a ±20.31	2488.44 ^c ±134.45	176.84 ^b ±4.92	198.46 ^c ±18.07
Group V	997.97 ^e ±9.34	4714.65 ^d ±640.28	262.79 ^d ±3.78	215.79 ^e ±7.69
Group VI	357.31 ^{bc} ±25.81	1852.35 ^a ±176.54	186.02 ^b ±13.42	77.26 ^a ±3.20

Values are given as mean ± SE of 8 animals. Values having different superscripts (a, b, c....) in a column are statistically different from one another at 5% level of significance.

areas were occasionally found (Fig 2b, 2d). Agaoglu *et al.* (2002) also found petechiae in the heart and focal muscle necrosis in goat heart after accidental intake of salinomycin in feed. Post mortem examination of heart in camels revealed pale and flabby heart and presence of congestive

heart failure after accidental salinomycin toxicity (Al-Wabel 2012). Similarly, Chen *et al.* (2014) also observed cardiomyocyte damage and heart failure after maduramicin toxicity and Shimshoni *et al.* (2014) also reported degenerative changes in the myocardium of pregnant gilts after toxicosis of maduramicin. Such type of changes in the heart may be due to ionic (Na^+ , K^+ or Ca^{2+}) imbalance caused by toxicity of ionophores since heart is more prone to ionic disturbances (Al-Wabel 2012). In heart of sheep, there was extensive subepicardial and myocardial hemorrhages, cardiomyolysis and myocarditis was seen by Ashrafihelan *et al.* (2014) due to accidental intake of salinomycin. Hemorrhages in heart were seen by Neeraja *et al.* (2004) in poultry birds on maduramicin toxicity and by giving ginseng and carotene their occurrence was reduced. Degeneration and necrosis were reported in the poultry hearts by Sadek *et al.* (2009) after salinomycin toxicity and addition of vitamin E or selenium diminished the severity of cardiac lesions. In our study also, vitamin C addition reduced the severity of heart lesions in intoxicated groups.

Table 6: DTH response as measured by variation in skin thickness (mm) in different groups using PHA-P as an allergen.

Treatment group	Before PHA-P skin thickness (mm)	After PHA-P (24 hour) skin thickness (mm)
Group I	0.10 $\pm$ 0.05	0.30 $\pm$ 0.06
Group II	0.12 $\pm$ 0.04	0.30 $\pm$ 0.04
Group III	0.10 $\pm$ 0.08	0.17 $\pm$ 0.03
Group IV	0.13 $\pm$ 0.05	0.22 $\pm$ 0.02
Group V	0.14 $\pm$ 0.06	0.15 $\pm$ 0.01
Group VI	0.15 $\pm$ 0.08	0.23 $\pm$ 0.01

Values are given as mean $\pm$ SE of 8 birds.

Values having different superscripts (a, b, c.....) in a column are statistically different from one another at 5% level of significance.

Table 7: Histological lesion scores of various organs in different groups.

Lesion	Organ	Experimental groups					
		I	II	III	IV	V	VI
Congestion	Heart	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.63 $\pm$ 0.18 ^c	0.75 $\pm$ 0.16 ^b	2.75 $\pm$ 0.25 ^c	0.37 $\pm$ 0.18 ^{ab}
	Skeletal Muscle	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.62 $\pm$ 0.26 ^c	0.75 $\pm$ 0.16 ^b	2.87 $\pm$ 0.12 ^c	0.25 $\pm$ 0.16 ^a
	Gizzard	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.12 $\pm$ 0.12 ^c	0.62 $\pm$ 0.18 ^b	2.00 $\pm$ 0.18 ^c	0.12 $\pm$ 0.12 ^a
	Liver	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.25 $\pm$ 0.16 ^c	0.75 $\pm$ 0.16 ^b	2.75 $\pm$ 0.16 ^d	0.62 $\pm$ 0.18 ^b
	Kidneys	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.12 $\pm$ 0.12 ^b	0.25 $\pm$ 0.16 ^a	2.5 $\pm$ 0.18 ^b	0.37 $\pm$ 0.18 ^a
	Spleen	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.37 $\pm$ 0.18 ^c	0.62 $\pm$ 0.18 ^b	2.62 $\pm$ 0.18 ^c	0.37 $\pm$ 0.18 ^{ab}
Hemorrhage	Heart	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.25 $\pm$ 0.25 ^b	0.37 $\pm$ 0.18 ^a	2.62 $\pm$ 0.18 ^b	0.37 $\pm$ 0.18 ^a
	Skeletal Muscle	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.50 $\pm$ 0.18 ^b	0.25 $\pm$ 0.16 ^a	2.25 $\pm$ 0.16 ^b	0.12 $\pm$ 0.12 ^a
	Liver	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	1.37 $\pm$ 0.18 ^b	0.12 $\pm$ 0.12 ^a	2.00 $\pm$ 0.27 ^c	0.00 $\pm$ 0.00 ^a
	Kidneys	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.12 $\pm$ 0.12 ^b	0.25 $\pm$ 0.16 ^a	2.12 $\pm$ 0.22 ^b	0.12 $\pm$ 0.12 ^a
Degeneration and necrosis	Heart	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.87 $\pm$ 0.12 ^c	0.75 $\pm$ 0.25 ^b	3.00 $\pm$ 0.00 ^c	0.37 $\pm$ 0.18 ^{ab}
	Skeletal Muscle	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.75 $\pm$ 0.16 ^c	0.50 $\pm$ 0.18 ^b	2.87 $\pm$ 0.12 ^c	0.25 $\pm$ 0.16 ^{ab}
	Gizzard	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.12 $\pm$ 0.12 ^c	0.37 $\pm$ 0.18 ^b	2.12 $\pm$ 0.12 ^c	0.12 $\pm$ 0.12 ^{ab}
	Liver	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.37 $\pm$ 0.18 ^c	0.62 $\pm$ 0.18 ^b	2.87 $\pm$ 0.12 ^d	0.50 $\pm$ 0.18 ^b
	Kidneys	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.12 $\pm$ 0.12 ^c	0.25 $\pm$ 0.16 ^{ab}	2.62 $\pm$ 0.18 ^d	0.5 $\pm$ 0.18 ^b
Inflammatory cells infiltration	Spleen	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.00 $\pm$ 0.00 ^c	0.37 $\pm$ 0.18 ^b	2.12 $\pm$ 0.12 ^c	0.50 $\pm$ 0.18 ^b
	Heart	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.62 $\pm$ 0.18 ^c	0.12 $\pm$ 0.12 ^a	3.00 $\pm$ 0.00 ^d	0.62 $\pm$ 0.18 ^b
	Skeletal Muscle	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.87 $\pm$ 0.12 ^b	0.12 $\pm$ 0.12 ^a	3.00 $\pm$ 0.00 ^b	0.12 $\pm$ 0.12 ^a
	Gizzard	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.00 $\pm$ 0.00 ^c	0.37 $\pm$ 0.18 ^b	2.00 $\pm$ 0.00 ^c	0.12 $\pm$ 0.12 ^{ab}
	Liver	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.62 $\pm$ 0.18 ^c	0.50 $\pm$ 0.18 ^b	2.50 $\pm$ 0.18 ^c	0.50 $\pm$ 0.18 ^b
Lymphoid depletion	Kidneys	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	1.75 $\pm$ 0.16 ^b	0.25 $\pm$ 0.16 ^a	2.12 $\pm$ 0.35 ^b	0.37 $\pm$ 0.18 ^a
	Spleen	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a	2.37 $\pm$ 0.18 ^b	0.37 $\pm$ 0.18 ^a	2.62 $\pm$ 0.18 ^b	0.25 $\pm$ 0.16 ^a

Values are given as mean $\pm$ SE of 8 birds. Values having different superscripts (a, b, c.....) in a row are statistically different from one another at 5% level of significance.

Skeletal muscles and gizzard

On gross examination, in group III and V, dryness and petechiae in the thigh muscles and breast muscles could be seen (Fig 1c). Pallor, wasting and loss of shine in muscles of birds of this group made them appear atrophied (Fig 1d) and especially ilio-tibialis was severely atrophied. Microscopically, there was severe degeneration and necrosis of skeletal muscles in group III and V characterized by widespread disruption and fragmentation of skeletal myofibers with loss of cross striations (Fig 2e, 2g). Expanding the interstitial space, eosinophilic fibrillar and karyorrhectic debris was seen interspersed between hyalinized myofibrillar fragments. Large aggregates of heterophilic infiltrates expanded perimysium and compressed eosinophilic degenerating and necrotic muscle fibers which were separated by interstitial edema (Fig 2e). In group IV and VI the muscles appeared taut with only mild intermuscular edema and myofiber degeneration (Fig 2f, 2h). In group III and V, gizzard musculature lacked normal glistening, seemed shrunken and atrophied. Histologically, examination of gizzard in ionophore alone administered groups revealed severe degeneration, necrosis of muscularis layer where the muscle fibers were replaced by fibrillar necrotic material and a heavy infiltration of inflammatory cells but mucosa was relatively spared. Intermuscular areas were expanded by dilated vessels with degenerating *tunica media* and infiltrate encompassing their serosa, perivascular edema, heterophilic and macrophage infiltration and fibroblastic proliferation. (Fig 2i, 2k). No obvious gross changes were

seen in gizzard muscles of group IV or VI group and histoarchitectural alterations were significantly less severe with only multifocal myo-degeneration predominantly vacuolation of sarcoplasm interspersed between healthy muscle fibers (Fig 2j, 2l). Extensive necrosis and fragmentation of muscle fibers in turkey birds due to accidental intake of salinomycin was seen by Assen (2006). Neeraja *et al.* (2004) also reported haemorrhage in thigh muscles after maduramicin toxicity. Diaz *et al.* (2018) observed cellular necrosis of pectoral, ilio-tibialis and breast muscle of birds due to salinomycin toxicity. Pallor of muscles, severe necrotic and degenerative myopathy with infiltration of neutrophils and macrophages in the muscles were seen in rabbits by Peixoto *et al.* (2009) because of intake of salinomycin. Likewise, Chen *et al.* (2014) found that maduramicin toxicity in birds caused skeletal muscle degeneration. Hyper eosinophilia, fragmentation, loss of cross striations and degenerative changes in the skeletal muscles in lactating sow was described due to salinomycin and maduramicin toxicity (Britzi *et al.* 2017). Similar to our results with vitamin C administration, Sadek *et al.* 2009, also found significant improvements in salinomycin induced pathomorphological alterations after vitamin E or selenium supplementation.

Liver and kidneys

In group III, severe congestion and hemorrhages were seen. Severe vacuolar degeneration and necrosis of hepatocytes with focal infiltration of inflammatory cells predominantly lymphocytes were seen (Fig 2m). In group IV, only congestion and mild degenerative changes and

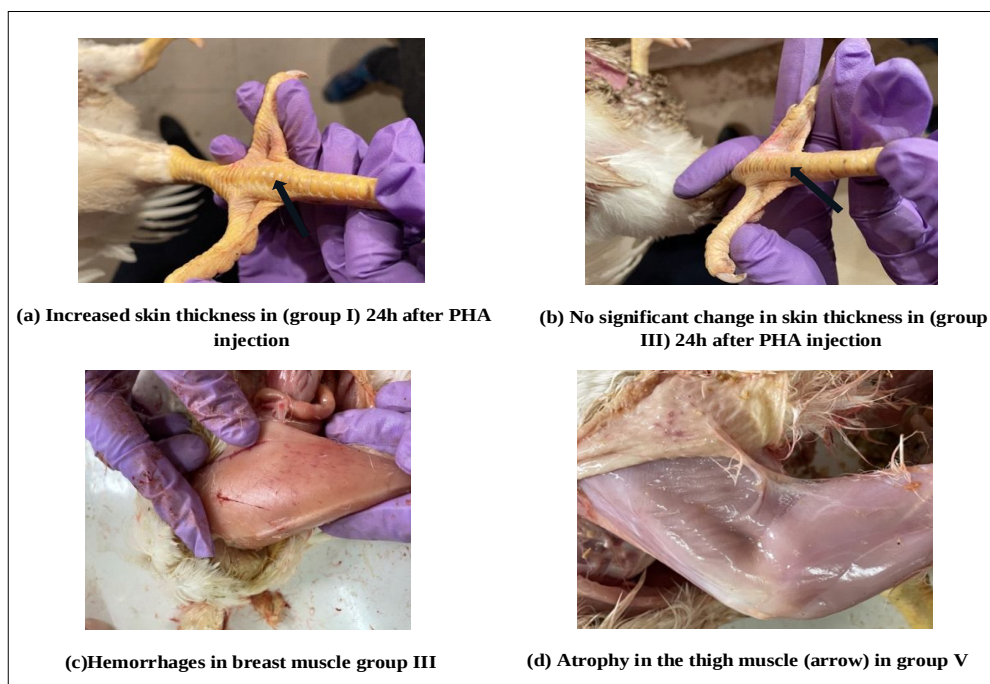


Fig 1: DTH response and salient gross lesions in muscles.

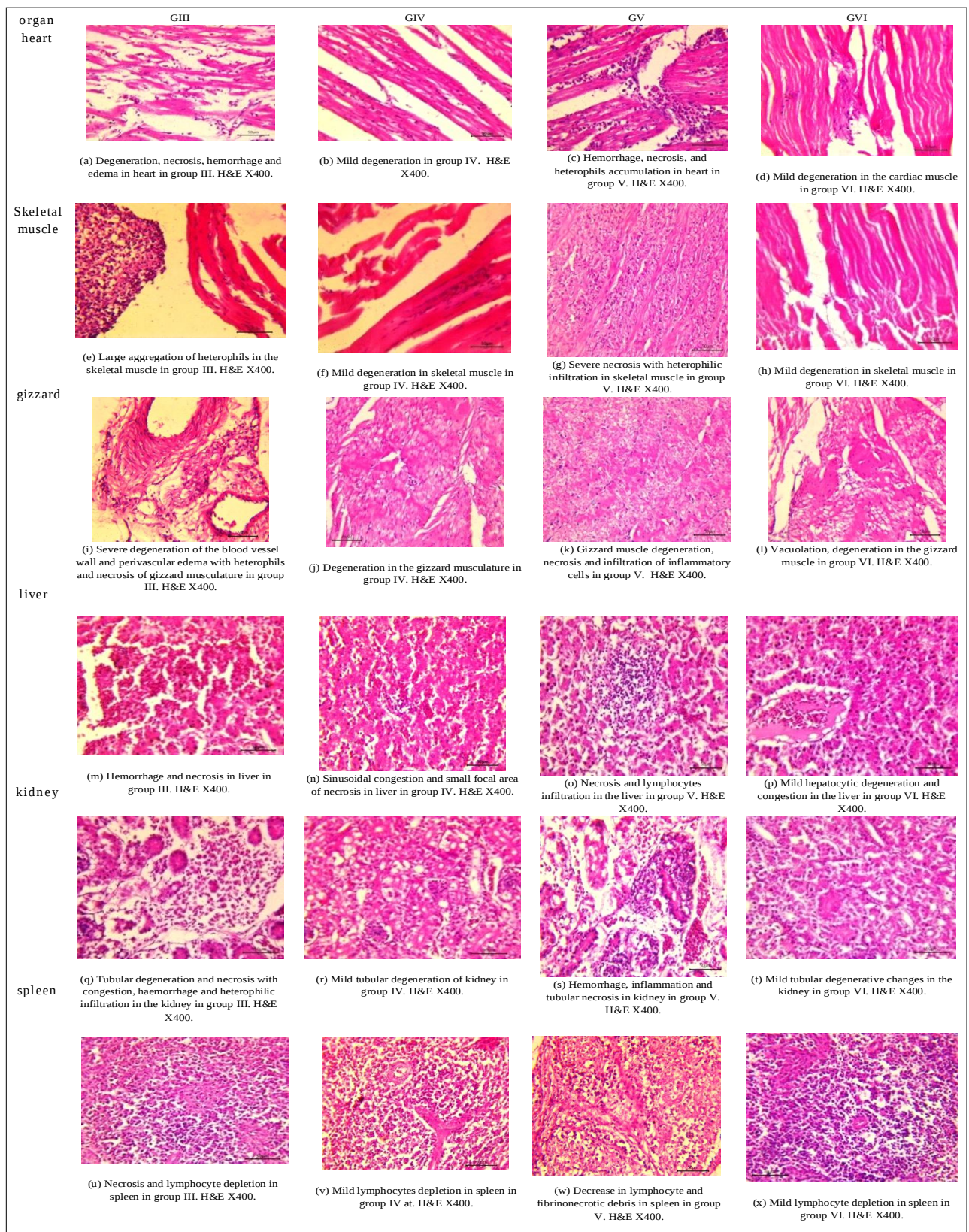


Fig 2: Histopathological lesions in heart, skeletal muscle, gizzard, liver, kidneys and spleen in groups III, VI, V and VI after 2nd WPE.

occasional focal necrotic areas could be appreciated (Fig 2n). In group V, severe lesions were seen which included congestion, hemorrhage, degeneration and necrosis. Necrotic areas were infiltrated by lymphocytes. In portal triad area, severe inflammatory changes were seen which included congestion, edema and inflammation (Fig 2o). In group VI, less severe changes were seen which included mild degeneration and congestion (Fig 2p). As far as kidneys were concerned, in group III birds there was tubular vacuolar degeneration along with focal necrosis of tubules. In the inter tubular areas in some birds, there was severe hemorrhage, congestion and edema (Fig 2q). In many areas, frank necrosis of tubules with hemorrhage and infiltration by heterophils was observed. In group IV, less severe changes consisting mostly of mild degeneration were seen. (Fig 2r). In group V birds, changes consisted of severe degeneration, necrosis of tubules and heterophilic infiltration (Fig 2s). In group VI, mild degenerative changes were seen in addition to mild congestion (Fig 2t). Similar changes in liver like enlargement, yellow discoloration and degeneration were also seen by Neeraja *et al.* (2004) after experimental maduramicin toxicity in birds. They also noticed degenerative changes in kidneys. Addition of the ginseng and carotene ameliorated these lesions. Rizvi *et al.* (2008) reported congestion of liver sinusoids and vacuolization of hepatocytes in broiler birds after experimental salinomycin toxicity. Koutoulis *et al.* (2013) found liver degeneration and swollen kidneys in broiler birds due to salinomycin toxicity. In camels, salinomycin toxicity led to hepatomegaly with multifocal hemorrhage and necrosis of hepatocytes and degeneration in renal tubules (Al-Wabel, 2012) which was similar to the findings of the present study. Ashrafihelan *et al.* (2014) reported focal necrosis, retention of bile and cholangitis in the liver and pale kidneys with extensive acute tubular necrosis in sheep due to accidental intoxication of salinomycin. In zebra fish, maduramicin toxicity caused vacuolar degeneration in the liver (Ni *et al.*, 2019). Sadek *et al.* (2009) found degeneration and necrosis in liver and kidney in birds intoxicated with salinomycin and usage of vitamin E or selenium reduced the degenerative lesions. In the present study, vitamin C was used to counteract effects of ionophore induced toxic changes and reduced the pathological changes in kidneys and Wong (2019) have also demonstrated that baicalein ameliorates cadmium induced oxidative hepato-renal damage.

Spleen

In group III, there was marked lymphocytic depletion in and around splenic arteriole and necrosis was also observed (Fig 2u). In group V, also lymphoid depletion and congestion was very pronounced (Fig 2w). Additionally, periarteriolar edema was observed. In contrast, in group IV and VI, lymphoid depletion was mild and no edema was seen (Fig 2v, Fig 2x). Similar findings have also been reported by Koutoulis *et al.* (2013), who saw splenic atrophy in broiler breeders and turkeys because of salinomycin toxicity.

CONCLUSION

The study showed that maduramicin and salinomycin caused significant toxicity in broiler chickens when added at 8 and 120 ppm in feed, respectively. Maduramicin and salinomycin reduced plasma total protein, albumin and globulin concentration, depressed immune responses and inflicted severe damage to antioxidant machinery as well as heart, gizzard and skeletal muscles in affected birds. Since ionophores are used recklessly under Indian field conditions, even marginal error in their addition in feed can cause reduced production and increased economic losses to farmers. Vitamin C could counter oxidative stress generated by feeding of ionophores and ameliorated free radical mediated pathological alterations in tissues as can also be seen by marked improvement in gross along with histological architecture of muscular tissues such as heart, skeletal muscle and gizzard. Vitamin C being cheap and easily available, can be supplemented in feed to confer significant protection against oxidative stress mediated ionophore toxicity in broiler chickens.

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Conflict of interest

The authors declare that there is no conflict of interest.

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