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TOXICOLOGICAL EVALUATION OF TILMICOSIN AFTER INTRAMUSCULAR INJECTION IN BROILER CHICKEN

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ABSTRACT

Cardiotoxic effects are more obvious with injectable dosage forms of tilmicosin. This study was designed to evaluate the toxicity of tilmicosin in broiler chicken after intramuscular injection. Seventy chicks were divided into seven groups. Group A was kept as control while groups B, C, D, E, F and G were administered tilmicosin at dose of 6.25, 12.5, 25, 50, 100 and 200mg/kg BW intramuscular for three days. Mortality was recorded. The birds treated with 6.25, 12.5, 25mg/kg of tilmicosin survived, while birds treated with 50, 100 and 200mg/kg died during first 30 minutes of injection. Serum samples were analyzed biochemically using spectrophotometer. A significant increase in levels of ALT, AST, total bilirubin, creatinine, blood urea nitrogen and decrease in levels of ALP, total protein and albumin was seen in groups treated with 12.5 and 25mg/kg of tilmicosin. Tilmicosin at 25mg/kg exhibited mild to severe lesions on liver, kidney and heart respectively. The birds treated with 50,100 and 200mg/kg of tilmicosin showed lesions on heart. Results were analyzed using two-way ANOVA followed by Bonferroni post analysis test. Tilmicosin elicited no significant alterations in biochemical and histopathological parameters at doses 6.25, 12.5mg/kg while mild alterations were observed at dose 25mg/kg. The 50, 100 and 200mg/kg doses of tilmicosin exhibited no change in the histopathological parameters of liver and kidney while changes were seen on heart. It is concluded that tilmicosin should be used intramuscular with care in therapy for not more than three days.

Keywords: Tilmicosin, toxicity, *in vivo*, biochemical, histopathological.

INTRODUCTION

Antibiotics have performed an overwhelmingly a significant role in staving off bacterial infections. Based on their mechanism of action antibiotics are classified into different groups namely penicillin, cephalosporins, tetracyclines, macrolide, aminoglycosides, and quinolones etc. Macrolides belongs to the group of antibiotics which are isolated from bacteria present in the soil i.e. genus *Streptomyces* (Riviere and Papich 2009). They are inhibitor of bacterial protein synthesis. Tilmicosin is a macrolide antibiotic prepared by chemical alteration of desmycosin and interferes with bacterial protein synthesis (Yazar *et al.* 2001). It is a broad spectrum antibiotic. It is available in injectable and feed premix mixture. It is usually recommended for prevention and treatment of pneumonia in sheep, pigs and cattle, associated with *Actinobacillus Pleuropneumoniae, Pasteurella multocida, Pasteurella haemolytica,* many species of Mycoplasma and many other microorganisms which are susceptible to tilmicosin. The dosage of tilmicosin as injectable in sheep and cattle is a single subcutaneous injection at the rate of 10 mg/kg body weight. In poultry the recommended dosage of tilmicosin is 10-20mg/kg bodyweight orally. It is widely used in Chronic Respiratory Disease (CRD), a disease is caused by *Mycoplasma gallisepticum* infection in chickens (Kempf *et al.,* 1997).

Veterinary drugs may be given in poultry birds individually or as a flock medication. In poultry, the convenient way of administering the drugs is oral on flock basis. The dosage of drugs that is administered in bird is based on body weight. The bird should receive the exact quantity of antibiotic agent which is required to treat the infections (Vermeulen *et al.* 2002). If the antibiotics are given by the methods other than parenteral, as via feed or water, the drug must be shared by other birds. It will not be possible to access that the bird had consumed the required therapeutic dose. With no recommended therapeutic dose, it not only cause risk in curing the bird, but there might be the chance for the development of resistance against that particular antibiotic, which is a huge and long term crisis. Further when the disease becomes more severe and there are chances of huge loss, the parenteral application provides a valuable substitute (Vermeulen *et al.* 2002). The most important benefit of the parenteral administration is the instant effects as a result of high blood and tissue levels reached within few hours post administration.

Tilmicosin principally affects the heart (Prescott and Baggot, 1993; Jordan *et al.* 1993). It produces tachycardia and decreased cardiac contractility in the heart after a single intravenous injection. It may cause

alteration in cardiac enzymes (Yazar *et al.* 2002a) and cardiac cell damage due to oxygen radicals and lipid per oxidation (Yazar *et al.* 2002b). Cardiotoxicity caused by tilmicosin in poultry is most obvious with injectable dosage forms (Prescott and Baggot 1993) however there are no published data to support this view. No data is available regarding the toxicity of tilmicosin under *in vivo* conditions particularly after intramuscular and intravenous administration in broiler chicken. The objective of this study was to evaluate the toxicity of tilmicosin after intramuscular injection in broiler chicken by performing biochemical and histopathological analysis of heart, liver and kidney.

MATERIALS AND METHODS

The research work was completed in the Department of Pharmacology and Toxicology, UVAS, Lahore.

Preparation of tilmicosin concentrations: The 20 grams of tilmicosin sodium phosphate powder was dissolved in 50 ml of distilled water (200mg/0.5ml). The dilutions i.e. 100, 50, 25, 12.5 and 6.25 were prepared by twofold serial dilution method from the stock solution (200mg/0.5ml).

Experimental Protocol: One day old broiler chicks (n=70) were brought from a local hatchery and were reared. On day thirty six, all the birds were randomly divided into seven groups having ten birds in each group. The birds in group A were administered normal saline at the dose of 0.2 ml/kg. The birds in group B, C, D, E, F and G were administered tilmicosin at the dose rate of 6.25, 12.5, 25, 50, 100 and 200mg/kg body weight respectively. The injections were given daily intramuscularly in the pectoral muscles for three consecutive days (Ramzan *et al,* 2015).

Blood Sampling and serological examination: Two ml of blood sample from every bird was collected after 24 hours (Day 1st) of first administration. Then after blood collection all the groups were administered second dose of different concentrations of tilmicosin in respective groups and again blood samples were collected after another 24 hours (Day 2nd). The same was repeated and blood samples were again collected after another 24 hours (Day 3rd). Serum was collected and stored at -20^{0}C till for further analysis. Serum samples were biochemically analyzed for the level of ALT, AST as described by (Wallhoffer *et al*. 1980), total bilirubin and total protein (Doumas *et al.* 1981). Albumin, creatinine and serum blood urea were analyzed by (Tietz 1999) on spectrophotometer (Photometer 5010 Semi-automatic, single-beam filter photometer, Berlin Germany).

Mortality: Mortality in groups, administered with different doses of tilmicosin was recorded till the completion of the experiment. Postmortem examination of birds was carried out in birds died during the experimental period and slaughtering of birds who remained alive till the completion of experiment. Tissue samples including heart, liver and kidney were collected for histopathological examination.

Histopathology: Tissue samples including heart, liver and kidney were collected. Necessary steps for processing of tissue for microscopic examination were performed including dehydration, clearing and embedding. Rehydration of tissues was done with alcohol and staining was then done with H&E stains as described by (Bancroft and Steven 1990). The slides were examined under a light microscope for histopathological changes (Bancroft and Steven 1990).

RESULTS

Death count in different dosage groups were noted. All the birds in groups A,B, C and D, administered with normal saline, 6.25, 12.5 and 25mg/kg respectively were survived throughout the experiment, while all the birds in groups E, F and G, administered with 50, 100 and 200mg/kg respectively died during first 30 minutes of intramuscular injection.

Biochemical analysis: The serum ALT levels were significantly high ($p<0.05$) in group C, treated with 12.5mg/kg of tilmicosin on 3rd day and significantly high ($p<0.01$, $p<0.001$) in group D, treated with 25mg/kg of tilmicosin on day 2nd and 3rd of treatment compared with control group. The serum AST levels were significantly high ($p<0.05$) in group C treated with 12.5mg/kg of tilmicosin on 3rd day and significantly high ($p<0.001$) in group D, treated with 25mg/kg of tilmicosin on all three days of treatment compared with control group. A significant decrease ($p<0.05$) was seen in levels of serum ALP in group D, treated with 25mg/kg of tilmicosin on all three days compared with control group. Total bilirubin levels were significantly high ($p<0.05$) and total protein levels were significantly lowered ($p<0.05$) in group D, treated with 25mg/kg of tilmicosin on day 2nd and 3rd of treatment respectively compared with control group. The levels of albumin were significantly decreased ($p<0.05$) in group C, treated with 12.5mg/kg of tilmicosin on day 3rd and in group D, treated with 25mg/kg of tilmicosin ($p<0.01$, $p<0.001$) on all the three day post treatment respectively compared with control group. An increase ($p<0.01$) in serum creatinine was observed in group C treated with 12.5mg/kg of tilmicosin on 3rd day and in group D treated with 25mg/kg of tilmicosin on 2nd and 3rd day of treatment. A significant increase ($p<0.05$) in serum blood urea in group D, treated with 25mg/kg of

tilmicosin was recorded on day 2nd and 3rd post treatment, when compared with the control group (Table 1).

Histopathology: No histopathological lesions were observed in groups treated with 6.25 and 12.5mg/kg of tilmicosin on heart, liver and kidney. The group treated with 25mg/kg of tilmicosin showed lesions on liver, while groups treated with 50, 100 and 200mg/kg showed no lesions on liver (Figure 1). The groups treated with 25mg/kg of tilmicosin showed lesions on kidney, while groups treated with 50, 100 and 200mg/kg showed no lesions on kidney (Figure 2). The group treated with 25 and 50, 100 and 200mg/kg of tilmicosin showed lesions on heart (Figure 3).

Table 1. Serum levels of ALT, AST, ALP, total bilirubin, total protein, albumin, creatinine and blood urea in broiler chicken treated with normal saline, 6.25, 12.5, 25mg/kg of tilmicosin on 1st 2nd and 3rd day of treatment.

Days	Parameters	Group A Normal Saline 0.2 ml/kg	Group B 6.25mg/kg	Group C 12.5mg/kg	Group D 25mg/kg
Day 01	ALT (U/L)	10.3±1.0	10.6±1.0	11.1±1.9	11.7±1.3
	AST (U/L)	186±6.9	182.5±8.8	190.9±7.7	194.7±7.3*
	ALP (U/L)	209.9±9.3	212.5±8.50	208.2±7.10	197.6±10.40*
	Total Bilirubin (mg/dl)	0.35±0.04	0.37±0.05	0.41±0.06	0.40±0.08
	Total Protein (g/dl)	4.43±0.3	4.33±0.3	4.29±0.3	4.35±0.5
	Albumin (g/dl)	2.16±0.1	2.12±0.1	2.13±0.1	2.03±0.15*
	Creatinine (mg/dl)	0.59±0.17	0.61±0.11	0.60±0.19	0.67±0.25
	Blood Urea (mg/dl)	56.5±6.0	57.1±5.5	57.6±3.6	60.8±3.8
Day 02	ALT (U/L)	10.7±1.8	10.4±0.8	12.2±1.9	12.9±1.4**
	AST (U/L)	180±6.4	180.0±7.5	185.8±5.6	192.6±8.5***
	ALP (U/L)	207.1±8.6	204.0±9.5	206.8±9.9	194.6±9.29*
	Total bilirubin (mg/dl)	0.34±0.03	0.34±0.04	0.37±0.05	0.42±0.07**
	Total protein (g/dl)	4.41±0.2	4.32±0.2	4.25±0.2	4.02±0.30*
	Albumin (g/dl)	2.17±0.08	2.08±0.07	2.11±0.1	2.02±0.1**
	Creatinine (mg/dl)	0.57±0.1	0.67±0.13	0.73±0.12	0.81±0.18**
	Blood Urea (mg/dl)	55.1±5.0	59.0±4.9	59.8±3.8	61.0±4.2*
Day 3rd	ALT (U/L)	10.2±1.5	11.3±1.1	11.9±1.2*	12.9±1.2***
	AST (U/L)	182.2±5.8	180.4±4.0	191.6±6.0*	201.0±4.0***
	ALP (U/L)	205.5±7.5	201.5±10.4	199.7±9.01	193.1±11.42*
	Total bilirubin (mg/dl)	0.36±0.06	0.34±0.06	0.38±0.04	0.47±0.04***
	Total protein (g/dl)	4.49±0.18	4.41±0.2	4.42±0.2	4.01±0.29**
	Albumin (g/dl)	2.14±0.07	2.10±0.08	1.99±0.08*	1.94±0.07***
	Creatinine (mg/dl)	0.61±0.19	0.64±0.14	0.84±0.12*	0.87±0.18**
	Blood Urea (mg/dl)	58.3±3.7	61.5±3.5	58.2±3.4	63.8±4.0*

(* $p<0.05$, ** $p<0.01$, *** $p<0.001$) U/L = units per liter, ALT= Alanine aminotransferase, AST= Aspartate aminotransferase, ALP= Alkaline phosphatase

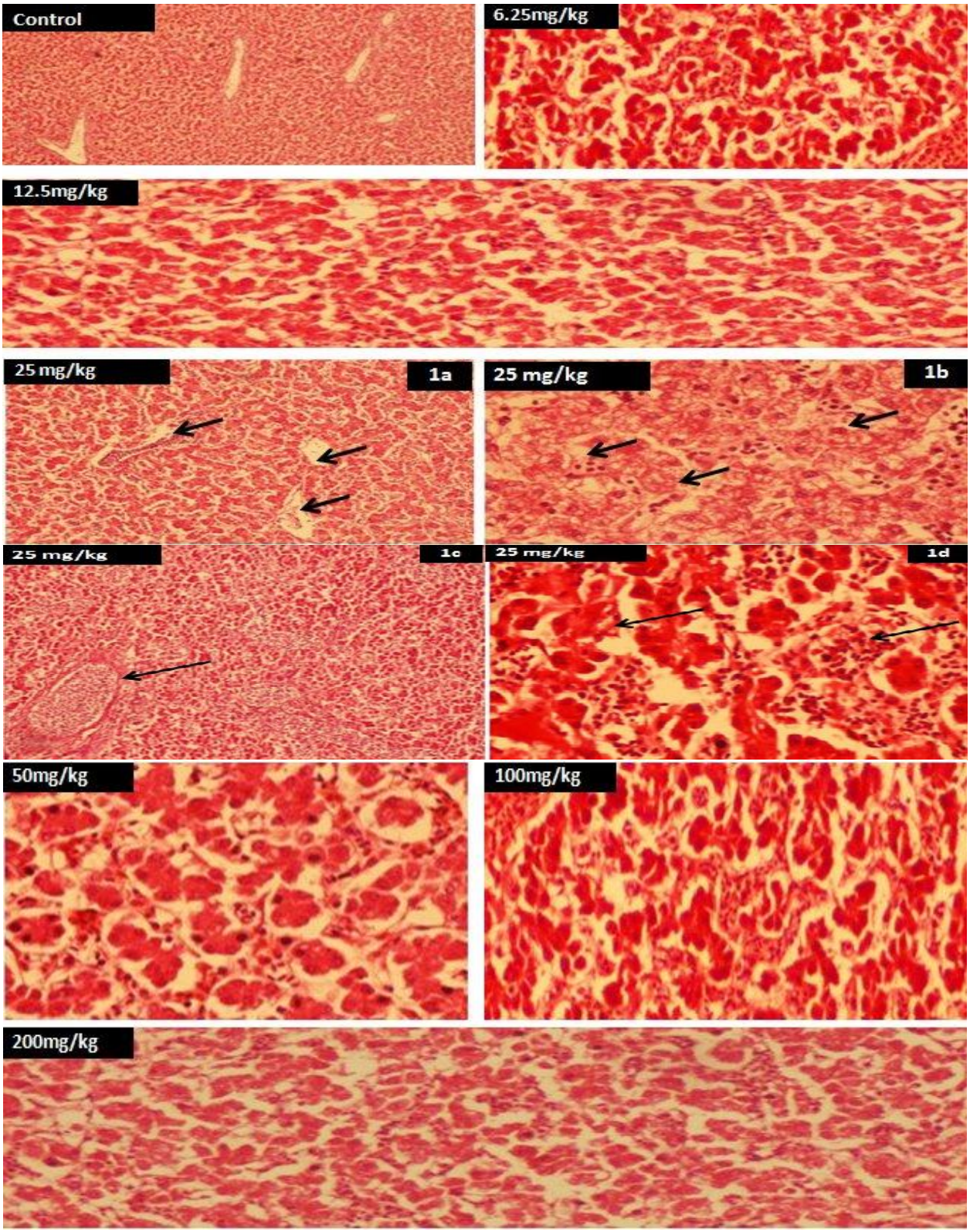

Figure 1. Histological sections of liver of chicken treated with 6.25, 12.5, 25, 50, 100 and 200mg/kg of tilmicosin. Dilatation of the central vein with diffuse cells proliferation (1a) and degenerative changes in hepatocytes (1b). Dilatation and congestion of the central vein (1c). Degeneration, focal necrosis in hepatocytes of hepatic cord (1d).

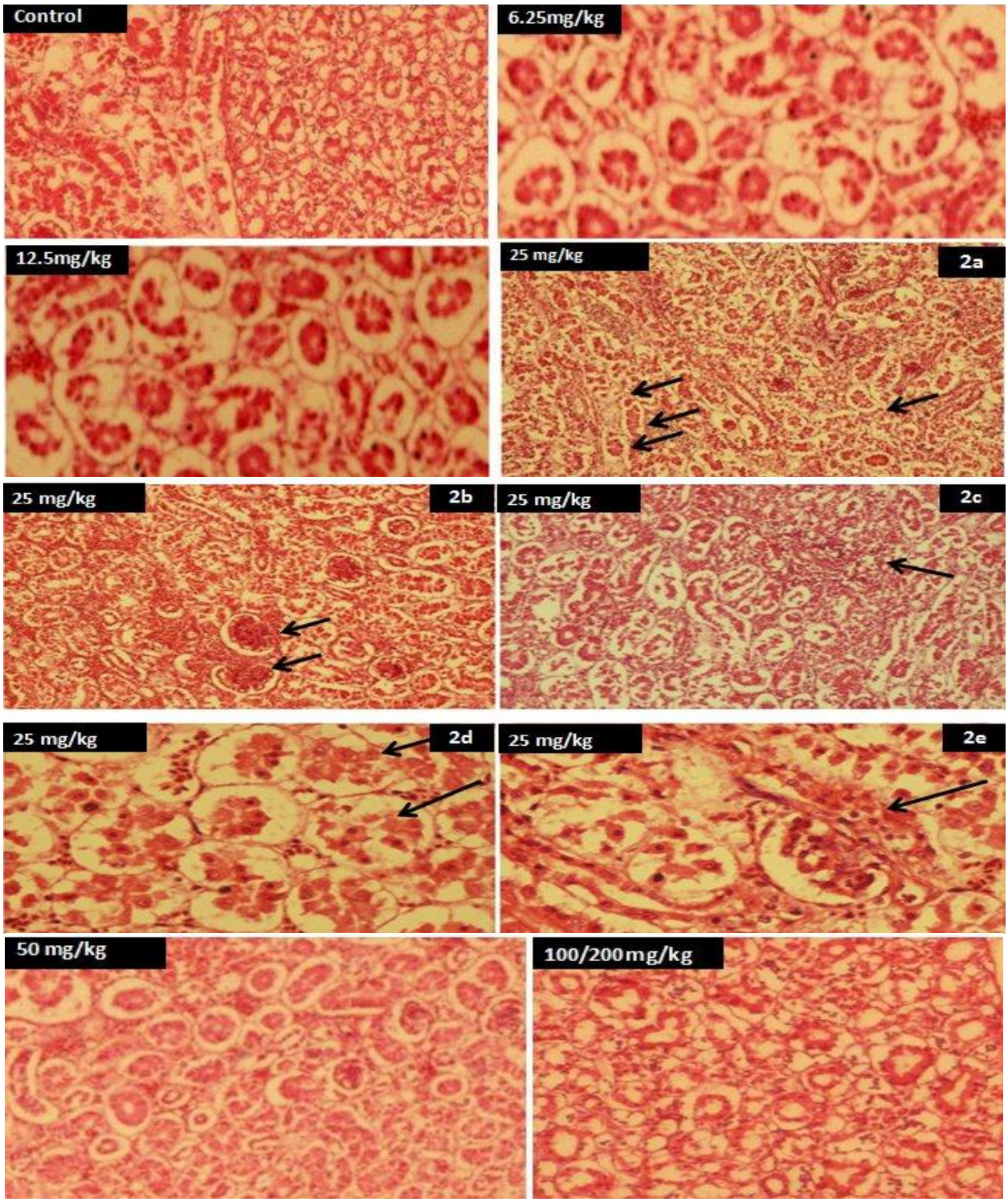

Figure 2. Histological section of kidney of chicken treated with 6.25, 12.5, 25, 50, 100 and 200mg/kg of tilmicosin. Infiltration of inflammatory cell in glomeruli and interstitial spaces, coagulative necrosis of renal epithelial cells in many tubular cells (2a). Hypertrophied glomerular (2b) with focal inflammatory cell infiltration in between glomeruli and renal tubules (2c). Destruction of proximal and distal convoluted tubules (2d&e).

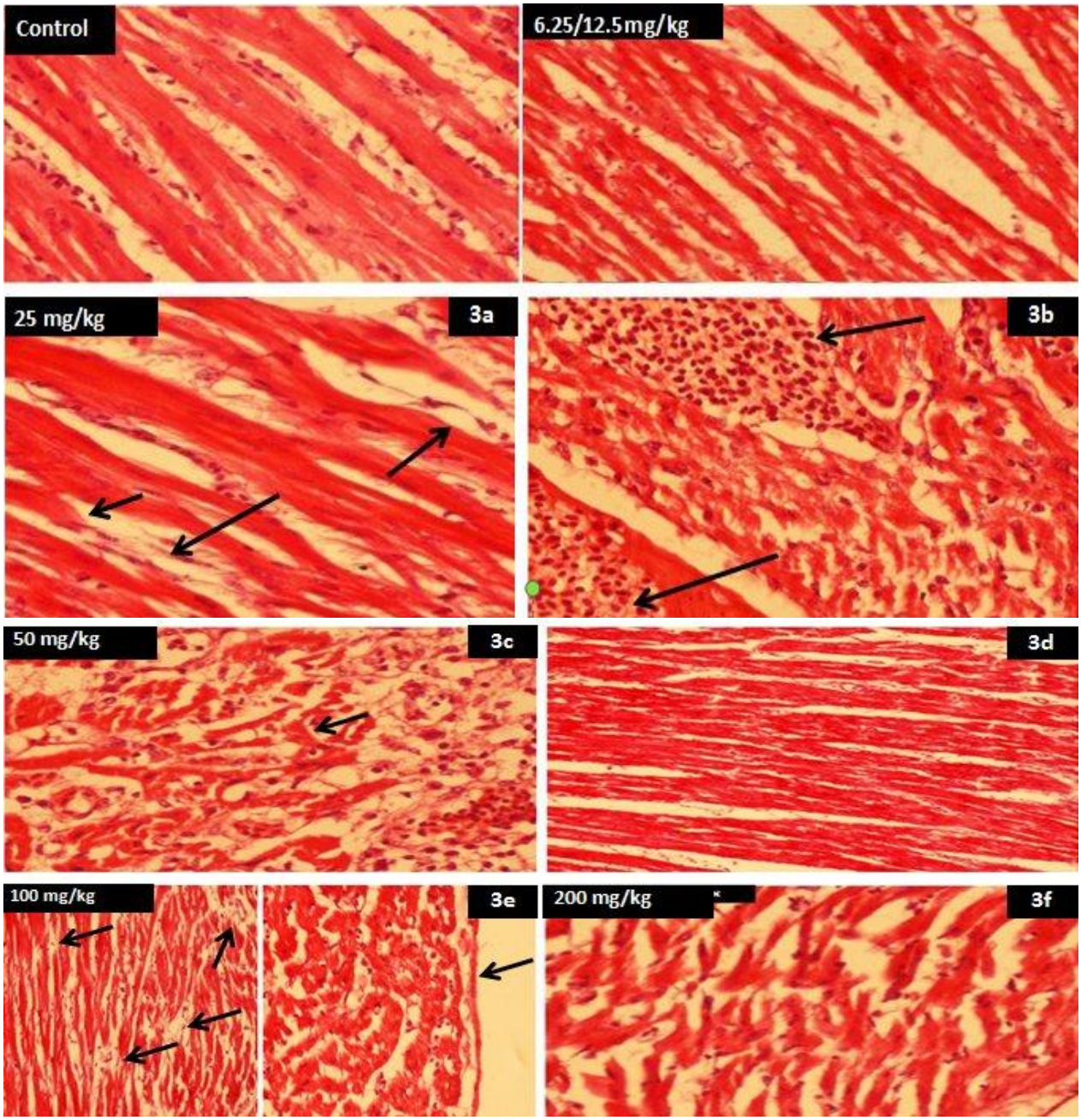

Figure 3. Histological section of heart of chicken treated with 6.25, 12.5, 25, 50,100 and 200mg/kg of tilmicosin. Mild degeneration in the cardiac muscles (3a). Mild congestion, engorgement of nucleated RBCs in blood vessels (3b). Degeneration in epi-cardial layer of heart, Congestion and inflammatory cells infiltrations were also seen (3c). Breakage areas observed in the myocardium (3d). Focal necrotic foci in myocardium, sloughing of basement and epi. Membrane (3e), breakdown of cardiac muscles, degenerative changes in myocardium (3f).

DISCUSSION

In this study different doses of tilmicosin were administered intramuscularly in broiler chicken for three successive days. All the birds survived which were administered normal saline, 6.25, 12.5 and 25mg/kg of tilmicosin. This is in accordance with (Xie *et al.* 2011) where mice survived at low dose. The present study indicated that the birds administered with 50, 100 and 200mg/kg intramuscularly died within 30 minutes of first injection. Xie *et al.* (2011) also observed similar type of response when tilmicosin was administered at higher

doses, all mice died within 2 hours. Gheith *et al.* (2015) results are also in accordance with the findings of present study. In the present study group B, treated with 6.25mg/kg of tilmicosin showed no significant increase in the activities of ALT. An increase in the levels of ALT was recorded in groups C and D, treated with tilmicosin at the rate of 12.5 and 25mg/kg respectively when compared with control. The results are in accordance with Gheith *et al.* (2015) who described a significant increase in ALT levels particularly post-administrating 40 mg/kg of tilmicosin in mice. Jordan (1987) observed high serum ALT after high dose of tilmicosin in dogs on day 12 and continued to increase till end of study. Xie *et al.* (2011) also described a significant increase in the levels of ALT in mice when treated with tilmicosin. Said *et al.* (2016) also seen significant increase in the levels of ALT in vaccinated and tilmicosin treated (10mg/kg subcutaneously) groups, when compared with control. Jordan (1992) and Altunok *et al.* (2002) stated that liver function parameters such as ALT were normal, suggesting that the damage of liver was slight and reversible and no tilmicosin treatment-related changes occurred. This might be due to single subcutaneous injection of tilmicosin at the dose of 25 mg/kg. Ali *et al.* (2012) also accessed serum ALT activities of clarithromycin suspension at the dose of 7.5 mg per kg and azithromycin at the dose of 12mg/kg. No change was observed in serum ALT activities. While tilmicosin in group administered with 12.5mg/kg produced slight increase in serum ALT activities only at 3rd day of the experiment.

In the present study group B, treated with 6.25mg/kg of tilmicosin showed no significant increase in the activities of AST. Ali *et al.* (2012) demonstrated similar type of results while evaluating the AST activities of clarithromycin suspension at the dose rate of 7.5 mg per kg and azithromycin at the dose of 12mg/kg. No change was observed in serum AST activities. The group treated with 12.5mg/kg of tilmicosin showed slight increase in the activities of AST on 3rd day of treatment. In this present study slight to significant increase in the activities of serum AST of groups treated with 25mg/kg was seen on all three days when compared with control. Gheith *et al.* (2015) described a significant increase in AST compared to control, particularly post-administrating 40 mg/kg of body weight dose in mice. Xie *et al.* (2011) described that AST levels were significantly increased in mice when treated with tilmicosin.

A decrease in the activities of serum ALP in group treated with 25mg/kg of tilmicosin on all the three days was recorded when compared with control. Fit *et al.* (2012) also seen similar type of effects by administering intramuscular injection of erythromycin into dogs. Jordan (1992) and Altunok *et al.* (2002) also stated similar type of results. Mossad *et al.* (2014) demonstrated that there was no change in ALP in chicken, which were administered 25mg/kg of tilmicosin orally. This might attributed due to difference in administration's route and rate of bioavailability.

The serum bilirubin levels of groups treated with 6.25 and 12.5mg/kg of tilmicosin were not significant on all three days when compared with control. While the group treated with 25mg/kg showed an increase in total bilirubin levels when compared with control. Altunok *et al.* (2002) had seen similar type of results in healthy New Zealand rabbits.

A significant decrease in the activities of total protein in group treated with 25mg/kg tilmicosin was recorded when compared with control. Mossad *et al.* (2014) found similar type of findings in broiler chicken. The results were also similar with Xie *et al.* (2011) where a significant decrease was observed in protein and albumin concentrations following subcutaneous injection of tilmicosin in mice. Kováčik *et al.* (2012) investigated and found that biochemical analysis of BHK-21 cells cultivated with tilmicosin showed a significant decrease in concentration total protein in almost all experimental groups.

In this study no change in albumin was recorded in group treated with 6.25mg/kg of tilmicosin while decrease in the activities of albumin was seen in groups treated with 12.5 and 25mg/kg of tilmicosin when compared with control. The results were in accordance with Er *et al.* (2011) who had seen no change in albumin concentrations while administering subcutaneous injection of tulathromycin at a dose of 10 mg/kg BW into rabbit. A decrease in activities of albumin in group treated with 12.5mg/kg on 3rd day was recorded. These obtained results were similar to the significant decrease in albumin concentrations following subcutaneous injection of tilmicosin into mice (Xie *et al.* 2011). Mossad *et al.* (2014) also observed low levels of albumin in serum of tilmicosin treated broiler chicken. Xie *et al.,* (2011) also produced similar type of results.

A significant increase was seen in levels of serum creatinine in groups treated with 12.5 and 25mg/kg tilmicosin when compared with control. Gheith *et al.* (2015) described a significant increase in creatinine, particularly post-administrating 40 mg/kg of tilmicosin in mice. Mossad *et al.* (2014) observed no change in levels of creatinine in broiler chicken treated with tilmicosin. This might be attributed due to difference in route of administration. Xie *et al.* (2011) also produced similar type of results. Ahmed *et al.* (2016) also observed no significant changes in creatinine levels. This may be attributed to difference in dose and route of administration. A slight increase in the activities of serum blood urea in group treated with 25mg/kg tilmicosin was recorded when compared with control. Gheith *et al.* (2015) described a significant increase in serum blood

urea, compared to control, particularly post-administrating 40 mg/kg of in mice.

Histopathology: The group treated with tilmicosin at the dose of 6.25 and 12.5mg/kg of tilmicosin showed no histopathological lesions on liver. While the group treated with 25mg/kg exhibited dilatation of central vein with diffuse cells proliferation and degenerative changes. There was slight swelling, proliferation between liver cells. There was dilatation and congestion of central vein, degeneration, focal necrosis in hepatocytes of hepatic cord. The results are in coordination with Gheith *et al.* (2015) where liver of mice treated with 20 and 40mg/kg of tilmicosin caused mild dilatation in central veins associated with diffused kupffer cells proliferation in between hepatocytes, focal necrosis of hepatic parenchyma associated with dilatation of the central veins. Further the groups treated with 100 and 200mg/kg showed no lesions in liver.

No histopathological changes were observed in groups treated with 6.25 and 12.5mg/kg tilmicosin on kidney. The group treated with 25mg/kg of tilmicosin showed infiltration of cell in glomeruli and interstitial spaces, coagulative necrosis of renal epithelial cells in many tubular cells, hypertrophied glomerular with focal inflammatory cell infiltration in between glomeruli and renal tubules and destruction of proximal and distal convoluted tubules. The results are also in accordance with Gheith *et al.* (2015) where focal inflammatory cell infiltration was detected in between the glomeruli and tubules at the cortex of the kidney of mice received small dose of tilmicosin. The larger dose of tilmicosin caused focal inflammatory cell infiltration in between the tubules and surrounding the congested blood vessels, associated with appearance of homogenous eosinophilic casts in the lumen of cystically dilated tubules. Further the groups treated with 50, 100 and 200mg/kg showed no lesions in kidney.

No histopathological changes were observed in groups treated with 6.25 and 12.5mg/kg tilmicosin on heart. The group treated with 25mg/kg of tilmicosin showed mild degeneration and congestion in cardiac muscles. The results are comparable with (Gheith *et al.* 2015) where degeneration, necrosis of cardiac cells and cardiac muscle fibers was seen in mice treated with 20mg/kg of tilmicosin. The group treated with 50mg/kg of tilmicosin showed degeneration in epi-cardial layer of heart, congestion and inflammatory cells infiltrations and breakage areas were observed in the myocardium. While the groups treated with higher doses of tilmicosin 100 and 200mg/kg of tilmicosin showed focal necrotic foci in myocardium, sloughing of basement and epi. membrane**,** breakdown of cardiac muscles and degenerative changes in myocardium. The results are also comparable with (Gheith *et al.* 2015). Tilmicosin caused temporary increase and decrease in serum levels of different liver enzymes. This increase or decrease was dose dependant. Death may be endorsed to sudden failure of the heart.

Conclusion: Tilmicosin has elicited no significant alterations in biochemical and histopathological parameters at dose 6.25, 12.5mg/kg while mild alterations were observed at dose 25mg/kg. The 50, 100 and 200mg/kg doses of tilmicosin exhibited no change in the histopathological parameters of liver and kidney while changes were seen on heart. It is concluded that tilmicosin should be used intramuscular with care in therapy for not more than three days.

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