

**An initial safety assessment of hepatotoxic and nephrotoxic potential of intramuscular ketoprofen at single repetitive dose level in broiler chickens**

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**ABSTRACT** A study was undertaken to assess the hepatotoxic and nephrotoxic potential of ketoprofen in comparison with diclofenac upon short-term intramuscular (i.m.) administration in broiler chickens. Eighteen broiler chickens were randomly divided into 3 groups of 6 birds each. Group I served as the control and received normal saline (0.1 mL, i.m.), group II was the positive control and received diclofenac sodium (2.5 mg/kg, i.m.), and group III received ketoprofen (3 mg/kg, i.m.) daily at 24-h intervals for 5 consecutive days. Diclofenac sodium-treated birds showed severe clinical signs of toxicity with high mortality, a significant increase ( $P < 0.01$ ) in serum concentrations of creatinine, uric acid,

alanine aminotransferase, and aspartate aminotransferase, and these changes correlated well with gross and microscopic examination findings of kidney and liver. In contrast, ketoprofen-treated birds did not show any adverse clinical signs and no significant increase in concentration of creatinine, uric acid, alanine aminotransferase, and aspartate aminotransferase when compared with birds in group I. Gross and microscopic examination of kidney and liver showed normal organ architecture. Thus, based on the present findings, it was concluded that ketoprofen at the dose of 3 mg/kg administered intramuscularly daily for 5 d was nontoxic to broiler chickens.

**Key words:** ketoprofen, diclofenac sodium, broiler chicken, toxicity

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**INTRODUCTION**

Prostaglandins are a few of many mediators that play a role in the augmentation of an inflammatory process and stimulate the production of vital compounds by a group of enzymes known as cyclo-oxygenases (COX). In chickens, the existence of COX and its broad tissue distribution was demonstrated by Yamada et al. (2006). Therefore, it seems reasonable to expect that, nonsteroidal antiinflammatory drugs (NSAID) can induce analgesia in birds. In the past, NSAID have been proposed in avian medicine for the treatment of a wide variety of clinical conditions (Bauck, 1990). For example, indomethacin was shown to have antiinflammatory effects in chickens (Ito and Bohn, 1986). The topical application of phenylbutazone to beak-trimmed chickens resulted in maintenance of feed intake (Glatz et al., 1992). Lame broiler chickens self-selected more feed containing an analgesic agent than sound birds (Danbury et al., 1997). Later, various experimental in-

flammation protocols were developed to determine the effectiveness of different NSAID in reducing articular pain in the domestic fowl (Hocking et al., 1997; Hocking et al., 2005). Treatment with carprofen was proven to increase the walking speed of affected chickens (McGeown et al., 1999).

Although NSAID were found to be effective in chickens for the treatment of a wide variety of clinical conditions, the dosage regimens instituted for treating many clinical conditions were often empirical or mostly extrapolated from other species. The extrapolation of drug dosages sometimes may lead to adverse effects (Dorrestein, 1992). For example, following a low dose of flunixin (0.1 mg/kg) administered parenterally for a short period to a Bobwhite quail resulted in renal damage (Klein et al., 1994). Renal ischemia and necrosis were reported in a Siberian crane following treatment with flunixin meglumine (5mg/kg; Paul-Murphy and Ludders, 2001). Furthermore, diclofenac was found to be nephrotoxic in poultry (Swetha et al., 2005; Prakash Reddy et al., 2006), and its toxicity was credited for a decline in the vulture population across the Indian subcontinent (Oaks et al., 2004).

The studies relating to diclofenac as a cause of vulture mortality caused the Drug Controller General of

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India to withdraw all licenses for the manufacturing of diclofenac intended for veterinary use in the year 2006. The ban on the use of diclofenac sodium has paved the way to find an alternative, safe NSAID for veterinary use. Ketoprofen, an aryl propionic acid derivative, has emerged as a good substitute for treating various clinical conditions of domestic animals. Besides, when the need arises, veterinarians or clinicians may use ketoprofen in chickens for the treatment of pain and other inflammatory conditions. Recent studies have found that ketoprofen, like diclofenac, causes renal tubular necrosis and visceral gout leading to mortality of male king eiders (*Somateria spectrabilis*) and male spectacled eiders (*Somateria fisheri*; Mulcahy et al., 2003), and more recently, ketoprofen-induced renal toxicity was observed in vultures (Naidoo et al., 2010).

There are very few reports relating to ketoprofen-induced toxicities in birds and there is no data pertaining to the safety of ketoprofen in chickens (*Gallus gallus*). Thus, information on the safety of ketoprofen in chickens will be important to veterinary practitioners. Hence, a study was designed with an objective to compare and assess the hepatotoxic and nephrotoxic potential of ketoprofen versus diclofenac upon short-term intramuscular administration in broiler chickens.

## MATERIALS AND METHODS

### Experimental Procedure

The Institutional Animal Ethics Committee (IAEC) approved (approval no. 29/LPM/IAEC/2009) the experimental protocol. Eighteen apparently healthy commercial broiler chickens (Cobb) aged between 5 to 6 wk with BW ranging from 1.8 to 2 kg were procured from a commercial poultry farm. They were maintained as per the protocol outlined in a publication of the committee for the purpose of control and supervision of experiments on animal's standard guidelines (CPCSEA, 2003). All birds were fed ad libitum a standard broiler feed (without antibiotics and coccidiostats) procured from the university poultry farm with free access to potable water. They were acclimatized to the laboratory housing conditions for a period of 7 d.

### Drugs

Voveran (injectable diclofenac sodium 25 mg/mL; Novartis, Mumbai, India) and Neoprofen (injectable ketoprofen 100 mg/mL; RFCL, New Delhi, India) were procured from a local pharmacy and used for the study.

### Dose Fixation

The ketoprofen dose of 3 mg/kg was based on available reports (Mulcahy et al., 2003; Mohan et al., 2008; Thompson, 2008), and this particular dose was chosen to assess its hepatotoxic and nephrotoxic potential in chickens. Diclofenac sodium is a proven nephrotoxic drug in birds and was used as a positive control, and

the dose (2.5 mg/kg) for chickens was based on a previous study conducted in our department (Swetha et al., 2005).

### Experimental Design

Eighteen broiler chickens were randomly divided into 3 groups of 6 birds each. Group I served as the control and received normal saline solution (0.1 mL, i.m.), group II was the positive control and received diclofenac sodium (2.5 mg/kg, i.m.), and group III received ketoprofen (3 mg/kg, i.m.) daily at 24-h intervals for 5 consecutive days. After drug administration, the birds were observed for onset and severity of clinical signs and mortality, if any, up to 5 d postadministration.

### Blood Sampling

Approximately, 1 mL of blood was collected through the cutaneous ulnar vein by using a disposable needle and syringe before treatment and subsequently on alternate days. The serum was separated and used for biochemical analysis.

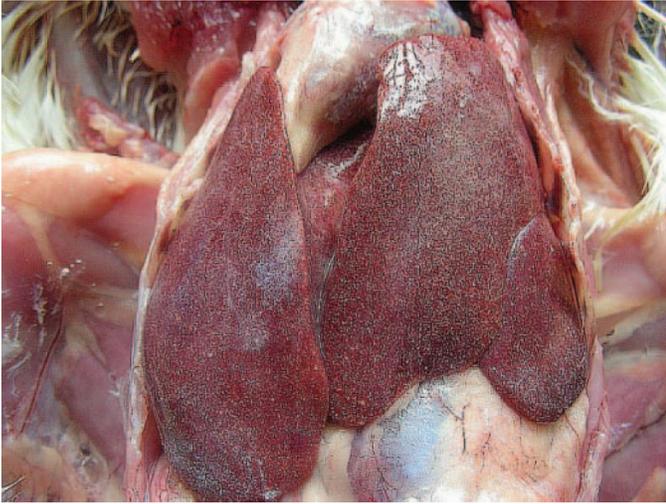
### Serum Biochemistry

The biochemical parameters measured included creatinine, uric acid, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) and were analyzed using an ARTOS semiautomatic biochemical analyzer (ONAN Biotech Pvt. Ltd., Secunderabad, Andhra Pradesh, India). Commercially available diagnostic kits purchased from M/s. Swemed Diagnostics, Bangalore, India were used. For specimen collection, all the birds that died during the course of the experiment and all the surviving birds at the end of experiment (on d 6) were killed by exsanguination and were subjected to a detailed pathological investigation. For histopathological examination, representative tissue samples from kidney and liver were collected in 10% neutral-buffered formalin as well as in absolute alcohol. The tissues fixed in neutral-buffered formalin were processed by a routine paraffin embedding technique, and sections 5 microns in thickness were cut and stained with hematoxylin and eosin (Luna, 1968). The tissues fixed in absolute alcohol were cleared and infiltrated with paraffin and sections were stained by DeGalantha's method to demonstrate urate crystals (Luna, 1968). The data was subjected to statistical analysis. Mean values and SEM were calculated and all the values were expressed as mean  $\pm$  SEM. The data was analyzed by one-way ANOVA followed by Dunnett's post hoc test using statistical software (GraphPad Prism, 2004), and  $P < 0.05$  was considered as significant.

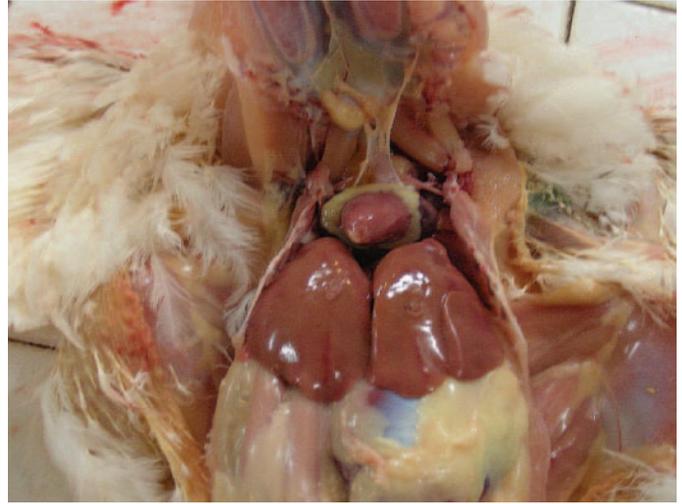
## RESULTS

### General Clinical Observations

In the present study, group I birds did not show any clinical signs of toxicity and were apparently healthy.



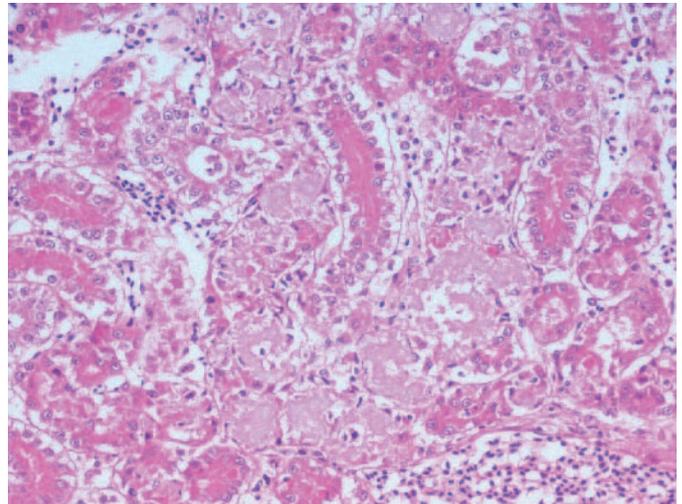
**Figure 1.** Group II-bird showing focal or diffuse urate deposits on the surface of the liver and heart. Color version available in the online PDF.



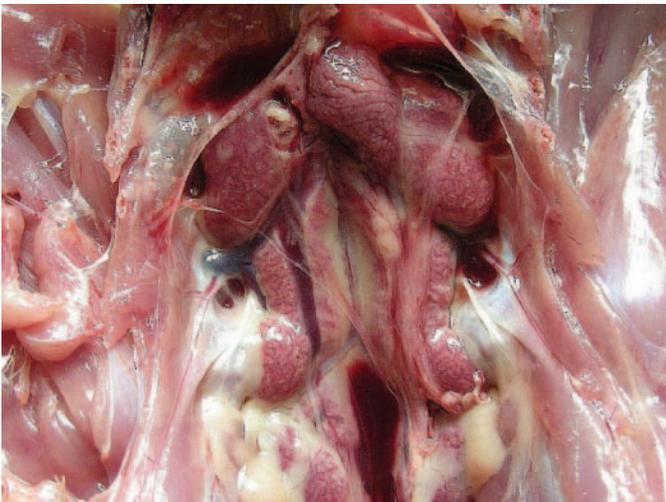
**Figure 4.** Group III-bird showing no apparent gross lesions. Color version available in the online PDF.



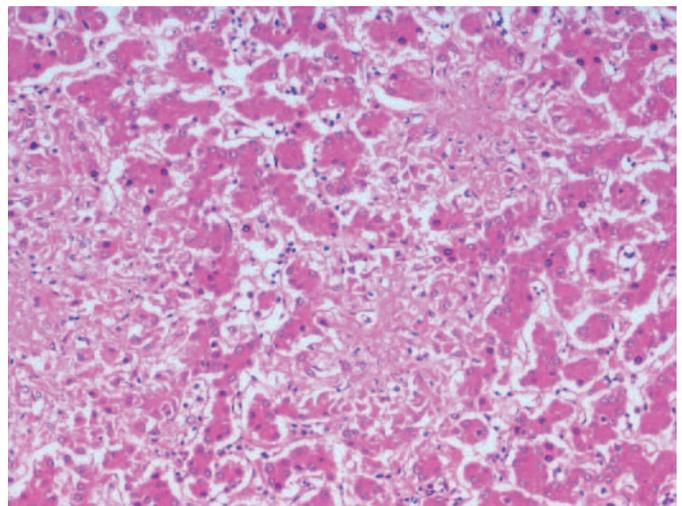
**Figure 2.** Group II-pericardium of heart showing considerable thickening with diffuse deposits of chalky white material. Color version available in the online PDF.



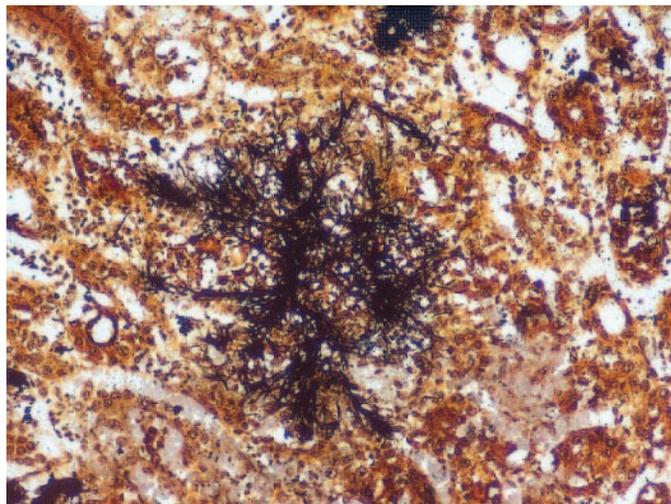
**Figure 5.** Section of kidney from group II, showing necrosis of tubular epithelium (hematoxylin and eosin  $\times 200$ ). Color version available in the online PDF.



**Figure 3.** Group II-bird showing enlarged kidneys with prominent lobulation. Color version available in the online PDF.



**Figure 6.** Section of liver from group II, showing hepatocyte degeneration and necrosis with radiating empty spaces indicative of urate deposits (hematoxylin and eosin  $\times 200$ ). Color version available in the online PDF.

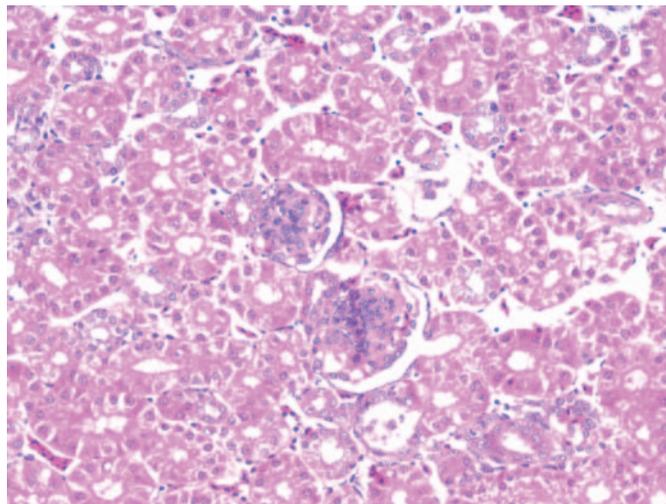


**Figure 7.** Section of kidney from group II, showing black-color-stained radiating urate crystal deposits in the kidney tubules (Degalanth's stain  $\times 200$ ). Color version available in the online PDF.

Group II birds showed clinical signs, such as dullness, anorexia, ruffled feathers, lethargy, depression, recumbence, shrunken eyes, and mucous mixed watery droppings, on d 3 and throughout the remainder of the experiment. Three out of 6 birds died in group II on d 3, 4, and 5, respectively. In contrast, group III birds, which received ketoprofen, remained apparently healthy throughout the experimental period.

### Biochemical Changes

Serum biochemical analysis revealed a significant increase ( $P < 0.01$ ) in values of creatinine, uric acid, ALT, and AST from d 3 onwards in the diclofenac sodium-treated birds when compared with the control group (Table 1). Whereas, the birds treated with ketoprofen did not show any significant change in serum

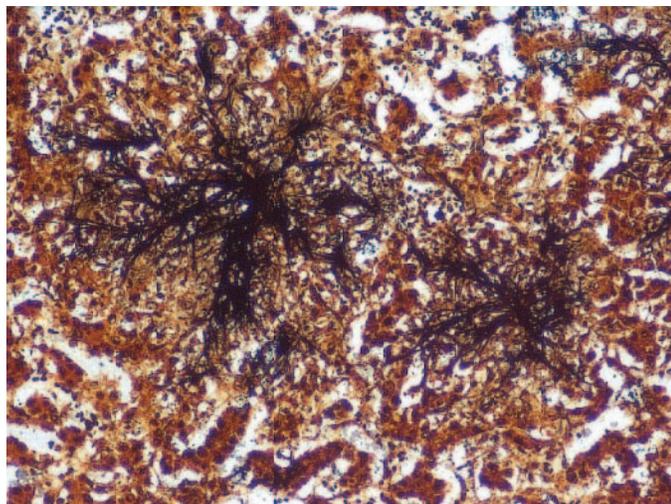


**Figure 9.** Section of kidney from group III, showing mild congestion and normal architecture of the kidney (hematoxylin and eosin  $\times 100$ ). Color version available in the online PDF.

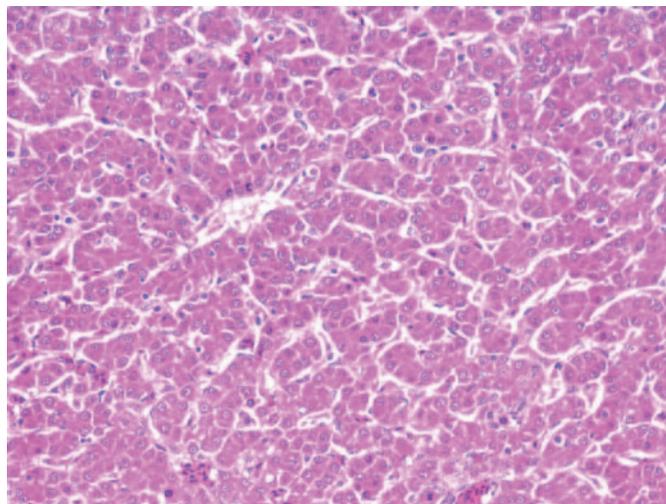
creatinine, uric acid, ALT, and AST concentrations when compared with the control group.

### Pathological Changes

At necropsy, group II birds showed extensive visceral gout characterized by chalky white deposits on the serosal surface of the heart and liver (Figure 1). Grossly, the pericardium of the heart revealed diffuse deposits of a chalky white material (Figure 2). The chalky white deposits on the serosal surface were confirmed to be uric acid by the Murexide reaction (Lumeij, 1994). The surface of the liver was congested and friable with deposits of a white material. The kidneys were whitish gray in appearance and were considerably enlarged, bulging out of the renal fossa and exhibiting prominent lobulations with multifocal chalky white urate crystal



**Figure 8.** Section of liver from group II, showing black-color-stained urate crystals arranged in a rosette pattern (Degalanth's stain  $\times 200$ ). Color version available in the online PDF.



**Figure 10.** Section of liver from group III, showing mild congestion of sinusoids and normal architecture of the liver (hematoxylin and eosin  $\times 100$ ). Color version available in the online PDF.

**Table 1.** Effect of nonsteroidal antiinflammatory drugs on biochemical parameters in broiler chickens<sup>1</sup>

Serum biochemical parameter	Group	Day		
		1	3	5
Creatinine (mg/dL)	Group I (Control)	0.38 ± 0.03	0.37 ± 0.03	0.42 ± 0.03
	Group II (Diclofenac sodium)	0.38 ± 0.03	0.73 ± 0.03**; n = 4	0.84 ± 0.02**; n = 3
	Group III (Ketoprofen)	0.40 ± 0.03	0.38 ± 0.01	0.41 ± 0.03
Uric acid (mg/dL)	Group I (Control)	2.55 ± 0.09	2.63 ± 0.06	2.83 ± 0.11
	Group II (Diclofenac sodium)	2.47 ± 0.13	6.36 ± 0.15**; n = 4	8.39 ± 0.55**; n = 3
	Group III (Ketoprofen)	2.62 ± 0.12	2.87 ± 0.01	2.61 ± 0.075
ALT <sup>2</sup> (U/L)	Group I (Control)	19.63 ± 0.29	19.87 ± 1.04	20.17 ± 0.82
	Group II (Diclofenac sodium)	16.21 ± 0.92	53.18 ± 3.20**; n = 4	64.43 ± 2.14**; n = 3
	Group III (Ketoprofen)	18.76 ± 2.89	21.99 ± 0.97	22.00 ± 2.99
AST <sup>3</sup> (U/L)	Group I (Control)	78.35 ± 4.83	82.60 ± 2.31	86.06 ± 2.57
	Group II (Diclofenac sodium)	75.87 ± 5.79	140.93 ± 5.03**; n = 4	211.43 ± 2.11**; n = 3
	Group III (Ketoprofen)	73.00 ± 4.33	85.3 ± 4.45	87.26 ± 3.71

<sup>1</sup>For each group, n = 6 unless otherwise noted. Values are means ± SEM.

<sup>2</sup>ALT = alanine aminotransferase.

<sup>3</sup>AST = aspartate aminotransferase.

\*\*P < 0.01 in relation to control.

deposits (Figure 3). In contrast, group III birds did not show any gross pathology (Figure 4).

Microscopic examination of kidney sections from the diclofenac-treated group showed focal or multifocal areas of tubular epithelial degeneration and urate deposits (tophi) either in the form of amorphous material or in a radiating crystalline pattern accompanied with necrotic debris (Figure 5). Liver sections showed congestion of sinusoids and vessels, hepatocyte degeneration, and necrosis accompanied with infiltrations of mononuclear cells (Figure 6). In addition, deposits of black-stained uric acid crystals in a rosette pattern with occasional focal irregular black spots were prominently observed in the DeGalantha's-stained sections of kidney and liver (Figures 7 and 8). However, group III birds did not show any histological changes either in the kidney or liver sections examined (Figures 9 and 10).

## DISCUSSION

Domestication of chickens was believed to occur in Southeast Asia. It has been reported that, the wild Red Jungle Fowl found in the forests of Southeast Asia spread to other parts of the world, resulting in the many chicken breeds of the present day (Stevens, 1991). Fumihito et al. (1996) concluded that 2 Red Jungle Fowl subspecies, *G. g. gallus* and *G. g. spadiceous*, are the direct ancestors of chickens. Thus, based on these historical reports, it was felt reasonable and logical to use broiler chickens in our experimental model. Moreover, the anatomy and physiology of these birds are almost identical to other chickens, they are comparatively cheaper, easy to procure and handle. Besides, housing and management practices are relatively simple.

The observed clinical signs of toxicity recorded in the diclofenac sodium-treated birds were in agreement with the findings of Swetha et al. (2005), wherein similar clinical signs of toxicity were observed in broiler chickens 24 h postadministration of diclofenac sodium (2.5 mg/kg i.m.). Swan et al. (2006) noticed lethargy

and different degrees of neck drooping in vultures, 24 h postoral administration of diclofenac sodium (2.5 mg/kg i.m.) and upon feeding of tissues from goats (*Capra aegagrus hircus*) and buffalos (*Bubalus bubalis*) treated with diclofenac a few hours before slaughter. Thus, the clinical signs of toxicity observed in group II were attributed to the toxic effect of diclofenac sodium on vital organs of birds.

Fifty percent of birds in group II died during the experimental period, confirming the renal toxicity induced by diclofenac sodium in birds injected intramuscularly with this drug. Similarly, high mortality was also reported in broiler chickens (Swetha et al., 2005), Vanaraja and PB1 poultry breeds (Prakash Reddy et al., 2006), and Leghorn layers (Naidoo et al., 2007) upon intramuscular administration of diclofenac sodium and also in vultures after oral administration of diclofenac sodium (Swan et al., 2006). The variation observed in the mortality rate of broiler chickens versus vultures may be due to the lower susceptibility of chickens to diclofenac sodium toxicity when compared with vultures. The cause of high mortality observed in the diclofenac sodium-treated birds was due to acute renal failure, resulting in hyperuricemia and visceral gout, which was evident upon postmortem and microscopic examination.

Most of the creatinine originates from the nonenzymatic conversion of creatinine in muscle. The creatinine thus produced is filtered by glomerular filtration. Creatinine after being filtered by the glomerulus is excreted in the urine. Because it is not excreted or absorbed by the renal tubules, it can be used as a rough index of glomerular filtration rate (Benjamin, 1985). The higher concentrations of serum creatinine observed in group II birds may be due to a blockade of renal vasodilatation due to nonselective inhibition of the COX by diclofenac sodium.

Diclofenac sodium causes a rare but potentially fatal hepatotoxicity that may be associated with the formation of reactive metabolites (benzoquinones imines) via

hepatic cytochrome P<sub>450</sub>-catalyzed oxidation and contributes to diclofenac-mediated hepatic injury (Tang et al., 1999). Thus, the higher concentrations of ALT and AST observed in group II birds are an indication of diclofenac-induced hepatocellular damage.

Furthermore, higher concentrations of creatinine along with ALT and AST are considered good indicators of muscle and liver damage. Creatinine, ALT, and AST concentrations were significantly higher for group II birds compared with the other groups, suggesting diclofenac-induced muscular and hepatocellular damage. It has been reported that the concentrations of ALT, AST, and creatinine shoot up in cases of hepatocellular and muscular damage (Lumeij, 1997). Elevated concentrations of serum ALT and AST, gross and histopathological changes in liver sections of group II birds were attributed to hepatic damage (Lumeij, 1999) due to the formation of reactive metabolites (benzoquinones imines).

The higher concentration of uric acid in serum observed in the present study following diclofenac administration may be due to renal failure, resulting in hyperuricemia and visceral gout. Furthermore, an increased serum uric acid concentration can be attributed to impaired uric acid excretion due to diclofenac-induced renal tubular degeneration, resulting in renal failure, leading to uric acid accumulation in blood and tissues and subsequent visceral gout and mortality.

Renal toxicities induced by NSAID are mainly due to inhibition of the COX1 enzyme. Changes include renal vasoconstriction and renal insufficiency (Boothe, 2001). Diclofenac is a powerful inhibitor of cyclo-oxygenases and prostaglandin synthetase, both involved in prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production (Vinals et al., 1997). The swollen kidneys with prominent lobulation and tubular necrosis may be attributed to excessive urate deposits in the tubules along with degenerative changes. Numerous large aggregates of amorphous urate material and cell debris with infiltration of inflammatory cells accompanied with loss of normal renal architecture were prominent in kidney sections of wild juvenile *Gyp* vultures upon experimental exposure to toxic concentrations of diclofenac (Meteyer et al., 2005). The black-colored uric acid crystals observed in the kidney sections of group II birds are indicative of visceral gout. In avian species, the excretion of uric acid takes place at the proximal renal convoluted tubules, a process considered to be energy-dependent (Siller, 1981; Goldstein and Skadhauge, 2000). When kidneys fail to remove uric acid efficiently from the blood, tissues become supersaturated with uric acid, resulting in urate salt precipitation as crystals (Lumeij, 1997). In the present study, deposition of black-colored radiating or amorphous uric acid material in the renal tubules of the diclofenac-treated birds are indicative of urate salt crystals.

Necrosis of the proximal renal convoluted tubules would compromise uric acid excretion, leading to a rapid elevation of uric acid concentration in the blood.

Once the saturation point is reached, uric acid would rapidly precipitate as crystals on the organ surface and within the organ parenchyma, resulting in death (Johnson, 1979). Deposits of uric acid crystals on vital organs, such as heart, liver, and kidneys in group II birds could be attributed to renal failure, resulting in visceral gout and death.

Renal tubular necrosis and visceral gout were reported in king eiders and spectacled eiders treated with ketoprofen (Mulcahy et al., 2003). In addition, necropsies of vultures that died from ketoprofen toxicity revealed gross morphological changes, characterized by severe nephrotoxicity with diffuse visceral gout (Naidoo et al., 2010). In contrast, in the present study, no gross or histological alterations were observed in kidneys or livers of broiler chickens treated with ketoprofen. Supporting these findings were the unaltered serum concentrations of creatinine, uric acid, ALT, and AST in the ketoprofen-treated birds, suggesting absence of toxic effects on kidneys or liver. In conclusion, our findings suggest that ketoprofen at the dose of 3 mg/kg when administered intramuscularly every 24 h for 5 d do not induce renal or hepato toxicity in chickens.

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