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EVALUATION OF DICLOFENAC SODIUM TOXICITY AT DIFFERENT CONCENTRATIONS IN RELATION TO TIME USING BROILER CHICKEN MODEL

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ABSTRACT

Toxicity of diclofenac sodium was evaluated in relation to clinical signs, post mortem findings, liver and kidneys functions in broiler chicken model. Broiler birds (35th day) of groups A1, A2, A3 and A4 were injected diclofenac sodium at dose rate of 1.25, 2.5, 5 and 10 mg/kg body weight intramuscularly, respectively for four consecutive days. The birds of group A5 were injected with 0.2 ml/kg of physiological normal saline. Same dosage regime was followed for groups B1, B2, B3, B4 and B5 using oral route. Clinical signs observed in birds receiving 2.5, 5 and 10 mg/kg body weight diclofenac sodium were lethargy, asleep, no response to external stimuli, closed eyes, deep breathing and difficulty while standing and walking. Percent mortality recorded in different treatment groups showed increasing fashion with higher dose of diclofenac sodium used by either route. The major gross lesion was visceral gout manifested as mild to severe and widespread deposition of a mixture of white chalky material (uric acid crystals), white debris (uric acid) and varying amounts of fibrinous exudate on all the visceral organs. Serum uric acid values at 24, 48, 72 and 96 hours post-exposure were significantly higher in same groups than control and groups treated with diclofenac sodium 1.25 mg/kg body weight. Mean ± SD values of serum creatinine concentrations showed significant rise in relation to time than normal birds. A significant increase in serum AST activity was observed in samples from the birds of groups A2, A3, A4, B2, B3 and B4 at 24, 48, 72 and 96 hours after dosing ($p<0.05$). Values of serum ALT and ALP showed similarly rising trend with increase in the dose of diclofenac sodium used by either routes. It was concluded that diclofenac sodium is not a safe drug at higher doses by both routes.

Key words: Diclofenac sodium, visceral gout, broiler chicken, mortality and clinical signs.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are a group of chemically heterogeneous compounds, which act by inhibiting the biosynthesis of prostaglandins (PGs). Therefore, the therapeutic efficacy and adverse effects of NSAIDs are attributable to their ability to inhibit prostaglandin formation (Simmons *et al*., 2004). NSAIDs are used as anti-inflammatory in the treatment of musculoskeletal disorders such as rheumatoid arthritis, osteoarthritis and ankylosing spondylitis (Lees, 2009). The major adverse effects include gastric or intestinal ulceration and bleeding. Other side effects reported are disturbances in platelets and changes in renal function (Boothe, 2001).

Diclofenac, a phenyl acetic acid derivative has good analgesic, antipyretic and anti-inflammatory activity. In addition, diclofenac appears to reduce the intracellular concentration of free arachidonate in leukocytes, perhaps by altering the release or uptake of the fatty acids (Reiss *et al*., (1978).

Postoperative inflammation after cataract extraction is treated with an ophthalmic solution of diclofenac. GI tract damage like ulceration, bleeding or perforation of the intestinal wall is the adverse effect of diclofenac in human beings. Elevated hepatic aminotransferase activity indicates the possibility of liver damage by this drug (Aydin, *et al*., 2003). Other adverse effects associated with diclofenac therapy include CNS effects, skin rashes, allergic reactions, fluid retention, edema and rarely the impairment of renal function. After the detailed investigations, the researchers came to recognize that diclofenac, a pharmaceutical product being used to treat the domestic animals for various indications was the major cause of the huge causalities in the vultures. With renal damage in birds, there can be an impairment of the normal excretion of uric acid from body, such that it starts crystallizing on the surfaces and inside the visceral organs resulting in visceral gout and ultimately death (Oaks *et al*., 2004; Shultz *et al*., 2004). Based on the observations of these studies, the regional governments, *i.e.* India, Pakistan and Nepal imposed a ban on the use of diclofenac in animals in 2008. To make the ban enforceable, there was a dire need to find the safe alternate NSAID for use in veterinary practice. Such replacement was necessary to avoid depriving the animal patients and practicing veterinarians of an efficacious NSAID. Hence, the present research project was designed with the objective to develop a surrogate model to study the toxicity of diclofenac using broiler chickens as an alternative to the vulture.

MATERIALS AND METHODS

Experimental design: One hundred, day old broiler chicks *(Gallus gallus domesticus)* were kept and handled according to the guidelines framed by the Animal Usage and Care Committee of the University.

On day thirty-five, broiler birds were randomly divided into two main groups, (A and B) each of which was further divided into five subgroups. The birds in subgroups A1, A2, A3 and A4 were treated with diclofenac sodium at the dose of 1.25, 2.5, 5 and 10 mg/kg body weight, respectively by intramuscular (route), once daily for four consecutive days. The birds in subgroup A5 were injected with 0.2 ml/kg of physiological normal saline (PNS) intramuscularly and served as controls. Same dosage regime was followed for subgroups B1, B2, B3, B4 and B5 using oral route.

Clinical parameters: The birds in all groups were examined twice daily for their feeding, drinking and the general appearance. Abnormality in their behavior like depression, change in body movement, posture, feed and water intake were recorded.

Mortality: Mortality rates in different dosage groups were observed and recorded throughout the experimental period and compared to evaluate the toxicity of the test drug.

Blood sampling: Two ml blood was collected from each bird using vacutainer tubes from the right jugular vein. Blood was collected before the start of experiment and then after every 24 hours up to 96 hours. The blood samples were centrifuged for 10 minutes at 1500 rpm, serum was separated in serum cups and stored at -20^{o}C for further analysis.

Postmortem examination: Postmortem examination was performed on the birds that died during the experimental period as soon as possible after their death and on all the birds that survived at the end of experiment. Gross lesions in the external and internal organs were observed and recorded.

Biochemical Analysis

Analysis of urates: The white substance accumulated in the pericardium was collected and analyzed via the Murexide Test. Urate deposits were mixed with nitric acid and dried over a flame. A drop of concentrated ammonia was added, a mauve color develop and confirmed the presence of uric acid (Lumeij, 1994).

Serum samples separated from the blood samples collected at different time intervals were analyzed for the levels of uric acid and creatinine, and the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). The following procedures were used for the estimation of afore-mentioned assays by Spectrophotometer.

Uric acid: Serum uric acid was measured by a colorimetric method (Fossati *et al*., 1980). Serum samples processed for uric acid estimation at wavelength 520 nm. The accuracy and reproducibility were monitored by using assayed Multi-Sera for low, normal and elevated uric acid values.

Creatinine: Serum creatinine was measured using a colorimetric method described by Bartels and Bohmer (1972). The creatinine concentration in serum samples was measured against standard solution at wavelength 492 nm

Aspartate aminotransferase (AST): The colorimetric method of Reitman and Frankel (1957) was used for the determination of serum aspartate aminotransferase activity. The enzyme activity was measured at wavelength 546 nm at 37^{o}C. The enzyme activity (U/L) in serum samples was obtained from the table showing the absorbance vs. activity, provided along with the Reagent Kit. Assayed Multi-Sera for low, normal and elevated were used for quality control.

Alanine aminotransferase (ALT): A colorimetric method (Reitman and Frankel, 1957) was used for the determination of serum aspartate aminotransferase activity. The enzyme activity (U/L) of serum samples was obtained from the table showing the absorbance vs. activity, supplied along with the Reagent Kit. Assayed Multi-Sera for low, normal and elevated were used for quality control.

Alkaline phosphatase (ALP): Serum alkaline phosphatase was measured by optimized standard colorimetric method (Rec. GSCC, 1972). The quality control was monitored by using assayed Multi-Sera.

Statistical Analysis: Mortality percentages in different groups were compared. The data regarding serum uric acid, creatinine, AST, ALT and ALP were analyzed through two way analysis of variance technique. The difference among various treatment groups was calculated using LSD ($p<0.05$) (Steel *et al.*, 1997). The data was analyzed by Prism 5.0 software.

RESULTS

Clinical Findings: The birds in all the groups were found clinically healthy. On an average, within 24 hours post-treatment, birds in subgroups, A3, A4, B2, B3, and B4 started showing the signs of toxicity.

The affected birds appeared lethargic, perched and isolated from rest of the group. They appeared to be asleep and had stopped eating and drinking. The birds initially responded to external stimuli such as touch and

noise and woke up for a while before becoming depressed again. The birds kept their eyes closed for most of the time and had slow but deep breathing. The affected birds were reluctant to move and would only move a few steps when forced and soon sat down again. It was also observed that these birds sit on their knees and seem to have great difficulty while standing and walking.

Mortality: Percent mortality recorded in different treatment groups showed increasing fashion with higher dose of diclofenac used by either route (Table, 1).

Table-1: Percentage mortality in broiler chickens treated with diclofenac via either the intramuscular (IM) or oral route

Treatment Groups	Routes			
	Sub-group	IM	Sub-group	Oral
Diclofenac sodium (1.25mg/kg)	A1	0	B1	0
Diclofenac sodium (2.5 mg/kg)	A2	30	B2	10
Diclofenac sodium (5 mg/kg)	A3	40	B3	30
Diclofenac sodium (10 mg/kg)	A4	70	B4	50
PNS: (Control)	A5	0	B5	0

All the birds that died had similar lesions. The major gross lesion was visceral gout manifested as mild to severe and widespread deposition of a mixture of white chalky material (uric acid crystals), white debris (uric acid) and varying amounts of fibrinous exudate on all the visceral organs. A tooth paste-like white material was found in the pericardial sac of affected birds just after death which rapidly solidified to material like powdered sugar when exposed to air. The extent and distribution of gout on internal organs varied among individuals. The precipitation of urates varied from multifocal to locally extensive areas present in the subcutaneous tissues, pectoral muscles, thigh muscles, air sacs, thoracic wall serosal surface, pericardium, epicardium, sternum, abdominal fat, abdominal wall serosal surface, serosal surface of liver, spleen, proventriculus, ventriculus, entire intestinal tract, kidneys, and articular surface within mandibular, hip and hock joints. Small uroliths were found in segments of urethra and urethral openings of the cloaca. The liver was friable and kidneys were pale-tan and swollen. Varied amounts of white debris and fibrin were scattered on most of the visceral serosa.

The killed birds showed mild to moderate swelling and pale discoloration of kidneys when examined at the end of experiment. Visceral gout was absent in these birds. No gross lesions were found in the birds of control group and the surviving birds, other than those mentioned above of the treatment groups at the time of necropsy.

Biochemical Analyses: Sera collected from broiler chickens of all the groups before the initiation of experiment and 24, 48, 72, and 96 hours after the exposure to diclofenac via the intramuscular (IM) and oral routes were analyzed for uric acid, creatinine, AST, ALT and ALP.

Serum Uric Acid: There were no significant differences among treatment groups in serum uric acid concentrations in samples collected before the start of experiment ($p>0.05$). Mean ± SD values of serum uric acid concentrations are shown in Table 2 for IM and oral routes, respectively. Uric acid values at 24 and 48 hours post-exposure from subgroups A2, A3 and A4 treated intramuscularly were significantly different from those of subgroup A5 (control). Similarly, subgroups A3 and A4 were significantly different from subgroup A5 at 72 hours. At 24 and 48 hours post-dosing the birds given diclofenac orally of groups B2, B2 and B2 were significant different from the control group (B5) whereas the uric acid concentrations in serum of groups B3 and B4 were significantly greater than controls at 72 hours after the first dose. No significant differences observed between serum uric acid concentrations for each dose for IM and oral routes at all time points except at 2.5 mg/kg at 48 hours post-exposure.

Serum Creatinine: Mean ± SD values of serum creatinine concentrations are depicted in Table 3. There was a significant rise in serum creatinine concentrations in samples from birds of group A3 when compared with those in the control group at 48 and 72 hours after the start of experiment. A significant increase was also observed in group A4 at 24, 48 and 72 hours ($p<0.05$). No significant difference from group A5 (control) was recorded in groups A1 and A2 at all the time points. In group B3, the serum creatinine concentration was significantly higher after 48 hours, and a significant rise was observed for group B4 after 24, 48, and 72 hours post-exposure. No significant difference was seen among groups in serum creatinine concentrations in samples collected at 0 hour.

Serum AST: A significant increase in serum AST activity was observed in samples from the birds of groups A2, A3 and A4 at 24, 48 and 72 hours after dosing ($p<0.05$). A similar rise was recorded in all the treatment groups compared to the control group at 96 hours. Groups B2 and B3 showed a significant rise in serum AST activity at 24 and 48 hours. The enzyme activity in group B4 was significantly higher at all the time points ($p<0.05$). There observed no significant difference in the enzyme activity among all the groups at the start of experiment. Mean ± SD values of serum AST are shown in Table 4.

Table-2: Serum uric acid concentrations (mg/dL) of broiler chickens treated with different doses of diclofenac via intramuscular (IM) and oral route administration (Mean ± SD) as a function of time

Group / Time	IM Route and Oral Route									
	A1 (1.25 mg/kg)	**A2 (2.5 mg/kg)**	**A3 (5 mg/kg)**	**A4 (10 mg/kg)**	**A5 (control)**	**B1 (1.25 mg/kg)**	**B2 (2.5 mg/kg)**	**B3 (5 mg/kg)**	**B4 (10 mg/kg)**	**B5 (control)**
0 hr	5.26± 0.42	4.6± 0.33	4.95± 0.5	5.08± 0.72	4.83± 0.51	4.86± 0.72	4.22± 0.4	4.78± 0.63	5.62± 0.36	5.23± 0.3
24 hr	6.32± 1.54	10.38± 3.25***	14.2± 5.37***	19.62± 7.66***	4.74± 0.96	5.94± 1.79	8.67± 2.55**	12.05± 4.57***	16.81± 6.5***	4.62± 0.25
48 hr	6.61± 1.37	15.6± 6.24***	20.67± 8.21***	24.22± 5.63***	4.92± 0.65	6.48± 1.68	11.44± 4.46***	17.8± 6.61***	19.31± 5.73***	4.94± 0.12
72 hr	6.72± 1.63	6.94± 1.03	11.78± 1.82***	14.45± 2.6***	5.15± 0.97	5.73± 1.32	6.87± 1.36	10.65± 1.96***	9.59± 3.7***	4.41± 0.36
96 hr	6.58± 1.42	6.87± 1.56	6.74± 1.78	7.32± 1.96	5.06± 0.83	6.36± 1.83	6.62± 1.25	6.74± 2.03	6.84± 1.46	5.06± 0.71

* p<0.05 *** p<0.001

Table-3: Serum creatinine concentrations (mg/dL) of broiler chickens treated with different doses of diclofenac via the intramuscular (IM) and oral route (Mean ± SD) as a function of time

Group / Time	IM Route and Oral Route									
	A1 (1.25 mg/kg)	**A2 (2.5 mg/kg)**	**A3 (5 mg/kg)**	**A4 (10 mg/kg)**	**A5 (control)**	**B1 (1.25 mg/kg)**	**B2 (2.5 mg/kg)**	**B3 (5 mg/kg)**	**B4 (10 mg/kg)**	**B5 (control)**
0 hr	0.37±0.03	0.39±0.07	0.38±0.06	0.37±0.03	0.39±0.05	0.39±0.05	0.37±0.06	0.38±0.04	0.39±0.05	0.38±0.03
24 hr	0.40±0.05	0.52±0.08	0.54±0.16	0.86±0.42***	0.38±0.04	0.42±0.04	0.43±0.04	0.46±0.09	0.76±0.24**	0.37±0.05
48 hr	0.42±0.05	0.64±0.32	0.99±0.86***	1.61±0.89***	0.38±0.05	0.40±0.07	0.52±0.12	0.78±0.64**	1.14±0.57***	0.39±0.03
72 hr	0.41±0.06	0.56±0.18	0.82±0.76*	0.92±0.13*	0.39±0.03	0.39±0.05	0.44±0.90	0.70±0.43	0.80±0.23*	0.38±0.06
96 hr	0.40±0.04	0.47±0.11	0.49±0.02	0.52±0.16	0.38±0.05	0.43±0.10	0.43±0.07	0.49±0.09	0.53±0.13	0.37±0.03

* p<0.05 *** p<0.01 *** p<0.001

Table-4: Serum AST activities (U/L) of broiler chickens treated with different doses of diclofenac via the intramuscular route (IM) and oral route (Mean ± SD) as a function of time

Group / Time	IM Route and Oral Route									
	A1 (1.25 mg/kg)	**A2 (2.5 mg/kg)**	**A3 (5 mg/kg)**	**A4 (10 mg/kg)**	**A5 (control)**	**B1 (1.25 mg/kg)**	**B2 (2.5 mg/kg)**	**B3 (5 mg/kg)**	**B4 (10 mg/kg)**	**B5 (control)**
0 hr	123.99± 13.57	125.12± 18.04	125.01± 17.11	127.62± 16.12	126.79± 14.95	129.31± 18.83	131.53± 12.44	120.72± 15.86	131.21± 17.23	123.52± 19.48
24 hr	137.06± 15.66	171.18± 35.10**	192.95± 43.30***	232.38± 48.44***	121.07± 11.15	130.06± 19.20	162.83± 33.64*	174.26± 28.12***	206.78± 39.64***	127.26± 13.58
48 hr	146.16± 17.65	197.29± 51.92***	227.97± 60.45***	250.98± 70.03***	126.21± 13.77	139.74± 25.28	183.64± 42.60***	197.64± 43.86***	242.35± 52.75***	120.62± 16.37
72 hr	155.42± 19.90	168.90± 42.52*	184.11± 34.44**	204.55± 37.40***	126.10± 15.07	142.63± 19.11	156.31± 25.69	159.67± 36.75	189.85± 33.80***	129.47± 14.72
96 hr	161.98± 16.10*	163.17± 24.55*	175.86± 29.12**	190.71± 29.98**	123.80± 16.81	154.63± 11.84*	143.58± 27.97	154.02± 27.32	172.24± 26.79**	122.93± 15.60

* p<0.05 ** p<0.01 *** p<0.001

Serum ALT: No significant difference was evident in serum ALT activities among the groups prior to initiation of the experiment. Mean ± SD values of serum ALT activities are presented in Table 5. In groups A2, A3 and A4, there was a significant rise in the enzyme activities at all the post-dosing sampling times when compared to t=0 ($p<0.05$). A similar pattern of rise in the ALT activity was manifested in groups B2, B3 at 24, 48, 72 and 96 hours after first dosing of the diclofenac.

Serum ALP: Serum ALP activities increased significantly in groups A2, A3, A4, B2, B3 and B4 after 24 and 48 hours ($p<0.05$). In groups B1 and B2, there was no significant rise in enzyme activities as compared to groups A1 and A2, respectively. No significant difference was observed among groups in pre-dosing serum ALP activities. Mean±SD values of serum ALP are shown in Table 6 for the IM and oral routes, respectively.

Table-5: Serum ALT Activities (U/L) of broiler chickens treated with different doses of diclofenac via the intramuscular (IM) and oral route (Mean ± SD) as a function of time.

Group / Time	IM Route and Oral Route									
	A1 (1.25 mg/kg)	A2 (2.5 mg/kg)	A3 (5 mg/kg)	A4 (10 mg/kg)	A5 (control)	B1 (1.25 mg/kg)	B2 (2.5 mg/kg)	B3 (5 mg/kg)	B4 (10 mg/kg)	B5 (control)
0 hr	23.43± 3.53	24.34± 2.62	24.71± 3.15	22.82± 4.96	23.06± 3.24	22.7± 5.75	25.16± 4.36	24.12± 4.43	21.64± 4.33	23.18± 5.07
24 hr	24.41± 4.02	33.32± 5.05***	54.58± 7.24***	63.33± 6.2***	22.26± 3.46	23.11± 4.86	29.53± 4.67*	48.25± 7.78***	56.74± 6.86***	21.79± 8.4
48 hr	26.1± 3.87	56.79± 7.44***	66.17± 6.7***	71.07± 9.09***	21.61± 5.84	25.08± 6.26	43.31± 5.24***	58.85± 8.32***	64.45± 8.43***	24.21± 7.37
72 hr	29.87± 3.32	45.18± 5***	54.78± 8.53***	58.88± 8.42***	25.15± 4.91	26.14± 4.75	37.8± 6.75***	46.67± 6.24***	51.23± 7.22***	22.85± 4.19
96 hr	32.72± 2.25***	36.63± 6.75***	42.36± 6.73***	47.52± 6.94***	23.84± 3.23	28.47± 7.06	30.29± 4.28*	37.25± 5.63***	42.14± 5.56***	21.59± 6.87

*** $p<0.001$ * $p<0.05$ *** $p<0.001$

Table-6: Serum ALP activities (U/L) of broiler chickens treated with different doses of diclofenac via the intramuscular (IM) and oral route (Mean ± SD) as a function of time

Group / Time	IM Route and Oral Route									
	A1 (1.25 mg/kg)	A2 (2.5 mg/kg)	A3 (5 mg/kg)	A4 (10 mg/kg)	A5 (control)	B1 (1.25 mg/kg)	B2 (2.5 mg/kg)	B3 (5 mg/kg)	B4 (10 mg/kg)	B5 (control)
0 hr	530.15± 29.87	524.05± 46.33	532.54± 37.18	526.17± 41.96	525.8± 43.02	527.47± 34.9	528.52± 28.53	522.12± 32.54	531.82± 38.03	532.21± 43.28
24 hr	545.41± 40.06	597.13± 48.44***	646.35± 44.56***	658.79± 40.52***	523.44± 38.12	541.2± 48.51	579.37± 43.9*	610.73± 42.91***	634.27± 49.23***	524.47± 23.31
48 hr	551.64± 45.81	632.06± 38.91***	685.58± 67.68***	691.82± 67.06***	526.88± 44.1	544.37± 37.42	606.42± 47.39***	642.7± 34.62***	653.55± 54.96***	529.05± 49.86
72 hr	550.16± 37.12	577.34± 43.1	550.11± 44.01	581.31± 46.77	527.93± 38.7	549.69± 40.76	558.25± 36.75	547.34± 28.94	566.2± 32.05	521.53± 39.69
96 hr	549.62± 40.1	554.1± 40.45	547.63± 32.9	553.05± 30.78	530.42± 29.65	542.5± 44.1	551.34± 33.85	546.08± 29.78	546.67± 35.08	534.07± 48.24

* $p<0.05$ ** $p<0.01$ *** $p<0.001$

DISCUSSION

Non-steroidal anti-inflammatory drugs (NSAIDs) are a group of chemically heterogeneous compounds used for more or less similar therapeutic indications. All NSAIDs, including selective COX-2 inhibitors, are antipyretic, analgesic, and anti-inflammatory, with the exception of acetaminophen, which is antipyretic and analgesic but is largely devoid of anti-inflammatory activity. Aspirin is the prototype NSAID which is the first drug of this group and still widely used despite development of newer and in some cases more potent compounds (Burke *et al.,* 2005). NSAIDs find their main clinical application as anti-inflammatory agents in the treatment of musculoskeletal disorders, such as rheumatoid arthritis and osteoarthritis (Chatterjee *et al.,* 1996). In general, NSAIDs provide only symptomatic relief from pain and inflammation associated with the disease, but do not arrest the progression of pathological injury to the tissue, and are not considered to be "disease-modifying" anti-rheumatic drugs.

NSAIDs are prescribed for pain of low to moderate intensity, such as dental pain. These drugs are also valuable in orthopedic and soft tissue surgical procedures, especially where extensive inflammation and

soft tissue trauma are present. Other clinical conditions in humans, where NSAIDs are prescribed include postoperative pain, dysmenorrhea, patent ductus arteriosus, systemic mastocytosis and cancer chemoprevention (Burke *et al*., 2005). In veterinary practice, these drugs are used to manage pain like postoperative pain, arthritis, and colic. NSAIDs are also used in animals as an adjunct therapy along with antimicrobial drugs to alleviate pain and inflammation (Lees, 2009).

Major adverse effects of NSAIDs observed in humans and other animals include gastrointestinal disorders, most notably mucosal damage, bleeding, ulceration, and perforation. Other adverse effects reported include nephrotoxicity, hepatotoxicity, blood clotting disorders and allergic reactions.

A unique toxic effect of diclofenac in vultures was reported when they ingested residues of the drug in carcasses of dead animals that had been treated shortly before death (Oaks *et al.*, 2004). It was observed that a sharp decline of at least three vulture species occurred in Southeast Asian countries including India, Pakistan, Nepal and Bangladesh (Prakash, 1999; Prakash *et al.*, 2001; Oaks *et al.*, 2004; Gilbert *et al.*, 2002). A rapid and massive decrease in vulture population was confirmed by the IUCN which declared three vulture species as 'critically endangered' (Green *et al.*, 2007). The decrease in the numbers of vultures has harmed the region's ecosystems and the environments. With vast reductions in vultures, other scavengers including stray dogs have increased. The increase in stray dogs, in turn, has increased risks of communicable diseases, including zoonoses. For example, the risk of rabies has greatly increased (Cunningham *et al.*, 2001; Prakash *et al.*, 2003). The experimental studies on vultures proved that *Gyps bengalensis* is highly sensitive to the toxicity of diclofenac. Indeed very low doses (0.8 mg/kg) are fatal to this species (Oaks *et al.*, 2004).

In order to find alternative NSAIDs to diclofenac, that are safer for scavenging birds and efficacious in target animals, a series of five experiments were designed in two phases. In the first phase, a chicken experimental model was developed to study the toxicity of various NSAIDs. Different drugs of this class were screened to establish toxicity profiles in comparison to diclofenac. In second phase, comparative efficacy studies of NSAIDs that were safer in chickens were conducted using target species under experimental and clinical situations in buffaloes and horses, respectively.

Keeping in view the non-availability of the vultures for experimental purpose, different bird species, including black kites, crows, pigeons, and broiler chickens were considered as potential experimental models. Broiler chickens were chosen due to easy availability, trouble-free handling and well studied physiological parameters.

Different doses of diclofenac sodium were administered to the birds of various groups intramuscularly or orally for four consecutive days. Twenty-four hours post-exposure, the birds in the higher dose groups, i.e. 5 mg/kg and 10 mg/kg body weight, started exhibiting clinical signs of mild to moderate depression followed by lethargy, anorexia, and reluctance to move. According to Lumeij (1994), in cases where renal urate deposition occurs prior to visceral gout, anorexia and lethargy may be noted for hours or days. Depression became severe with the passage of time and the affected birds stopped moving. In the advanced stages of toxicosis, the birds completely stopped eating and drinking, perched aside from the other birds, failed to hold their necks in normal posture, sat down with closed eyes, and ultimately died in the same position. Similarly, severe depression and lethargy characterized by drooping head syndrome had been observed in vultures, which were affected by the diclofenac toxicity either in the field or experimentally. These vultures were apparently unable to hold their necks in normal position (Prakash *et al.*, 2003; Swan *et al.*, 2005). Similar clinical findings from diclofenac toxicosis have also been reported in studies of experimentally dosed domestic fowl (Naidoo *et al.*, 2007). According to Speer (1997), birds experienced signs of lethargy, weakness and anorexia as a consequence of renal disease. Although the pH of the blood was not measured in this study, the development of depression in affected birds can likely be explained, at least in part, from metabolic acidosis, which may result from observed increases in serum uric acid, and this may be aggravated by reduced ability of proximal convoluted tubules to conserve bicarbonate (Seifter, 2004). Acidosis, similarly, leads to CNS depression in cattle and people (Blood and Radostits, 1989; Seifter, 2004). In this study, sudden death in the affected chickens was recorded. The acidosis developed due to increase in blood uric acid may result in hyperkalemia which might lead to cardiac arrest and the sudden death (Lierz, 2003).

The mortality ratios at different doses were significantly higher after intramuscular dosing compared to those treated via the oral route. This observation suggests that the bioavailability of diclofenac when administered orally may be lower than that of the intramuscular route. A 50% bioavailability of diclofenac was observed in fowl when given orally (Naidoo *et al.*, 2007). In the present study mortality rates of 40% and 30% were recorded at 5 mg/kg following intramuscular and oral administration, respectively. In two separate studies 33% and 40% mortality rates were reported with diclofenac at the same dose in layer and broiler chicken, respectively (Reddy *et al.*, 2006; Naidoo *et al*, 2007). In present study, no mortality occurred in broiler chickens given diclofenac at 1.25 mg/kg, whilst 100% mortality was reported in *Gyps bengalensis* vultures at the doses as low as 0.8 mg/kg (Oaks *et al.*, 2004). Vultures clearly

seem to be more sensitive to diclofenac toxicity than broiler chickens. This difference in susceptibility may be due to differences between the species in fate or receptor activity.

Postmortem examination conducted on the chickens that died due to diclofenac toxicity showed visceral gout as the most consistent postmortem lesion. It was distinctly evident in almost all the dead chickens of the various groups. Visceral gout was also observed as the main postmortem lesion in dead vultures collected from the wild and in those treated with diclofenac either orally or through meat of diclofenac treated animals (Gilbert *et al.,* 2002; Cunningham *et al.,* 2003; Oaks *et al.,* 2004; Swan *et al.,* 2005). A strong correlation was established in the presence of visceral gout and detection of diclofenac residues in body tissues of the affected vultures (Oaks *et al.,* 2004; Shultz *et al*., 2004). Urate crystals were extensively precipitated on the surface and within organ parenchyma in vultures with renal failure. (Meteyer *et al*., 2005). Visceral gout was also evident in a study of domestic fowl treated with diclofenac (Naidoo *et al.,* 2007). It is suggested that damage to the kidney tissue with diclofenac may interfere the normal excretion of uric acid from blood resulting in hyperuricemia. The excessive uric acid ultimately starts depositing inside and on the visceral organs resulting in the condition known as visceral gout (Lumeij, 1994). It has been reported that high dietary protein and calcium intake in birds may also cause hyperuricemia (Lumeij, 1994). Other studies showed that sodium bicarbonate can cause gout in broiler and layer chickens (Mubarak and Sharkawy, 1999; Ejaz *et al.,* 2005).

Excessive protein, calcium and sodium bicarbonate would not explain the observed development of visceral gout the present study because the same diet was fed to all the treatment as well as control groups, and visceral gout was not observed in the birds of the control groups.

Crespo and Shivaprasad (2003) stated that visceral gout is caused by renal failure. The kidneys may fail as a result of metabolic, infectious, or toxicologic diseases. Other lesions observed during necropsy included swollen and pale kidneys and friable liver. The nonsteroidal anti-inflammatory agent flunixin has been reported to cause nephrotoxicity in cranes and flamingos as well as gout in northern bobwhite quail [*Colinus virginianus*] (Klein *et al*., 1994). A significant rise in serum uric acid concentrations were observed with higher doses of diclofenac by 24 hours post-exposure whether dosing was via the intramuscular or oral routes. These findings are congruent with those reported from studies of *Gyps* vultures (Swan *et al.,* 2005). Similar observations were noticed by Naidoo *et al.* (2007) in domestic fowl after the treatment with diclofenac. By contrast no changes in uric acid concentration were observed in budgerigars (Pereira and Werther, 2007) and northern bobwhite quails (Klein *et al.,* 1994) treated with flunixin meglumine. This difference in observations may be due to different species and the variable sensitivity to the drug used. Differing explanations for increases in uric acid concentration in response to kidney damage have been made. The normal levels of uric acid were observed in renal damage in certain patients (Hochleithner, 1994) and as a consequence of the large volume of uric acid, that excreted from the tubules independently of the glomerular filtration rate, the concentration of uric acid in the blood did not easily change (Styles and Phalen, 1998) by contrast Fudge (1997) noted that an increase in blood uric acid concentration is indicative of renal disease and, if moderate to high increases are seen, there may be a significant tubular injury.

The findings of this study are fully supported by the results of a recent study in which hyperuricaemia was observed at 24 hours after treatment with both low and high dosages of diclofenac in oriental white-backed vultures [*Gyps bengalensis*] (Oaks *et al.,* 2004).

Serum creatinine concentrations provide another indicator of renal function. In this study, the higher doses of diclofenac administered to broiler chickens via both IM and oral routes were consistently followed by significant increases in serum creatinine concentrations.

These findings are completely in agreement with the observations made by Reddy *et al.* (2006) in broiler chickens after treatment with diclofenac. The findings of present study are also in line with those reported by Mathews, *et al.* (1990), which included significant elevations in serum creatinine concentrations in dogs given flunixin meglumine along with methoxyflurane.

In the current study of chickens it was observed that serum AST, ALT and ALP activities were significantly elevated, and this rise in enzyme activity was dose-dependent. The results of this study are in concordance with those reported by Swan *et al.* (2005) who observed a significant rise in ALT activity in *Gyps benegalensis* post-exposure to diclofenac. The findings of present study are also in harmony with the observations made in chickens treated with diclofenac (Reddy *et al.,* 2006). They noticed that the values of serum ALP and AST were significantly elevated in the diclofenac-treated group. A significant rise in ALT was also observed in dogs treated with flunixin meglumine (Mathews *et al.,* 1996), hence these observations are in line with the findings of the present study.

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