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Nephrotoxicity due to diclofenac alone and under the influence of certain variables in broilers

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Diclofenac was a widely used veterinary drug (now licenses have been withdrawn) in the Indian sub-continent for the management of inflammation, fever and/or pain in livestock. Vultures are exposed to diclofenac when scavenging on livestock treated with drug shortly before death (Swan *et al*. 2006). *Gyps* vultures in South Asia were considered among the most common large raptors in the world in mid-1980s (Houston 1985), but by 2000 three species, namely oriental white-backed vultures (*Gyps bengalensis*), long-billed vultures (*Gyps indicus*) and slender-billed vultures (*Gyps tenuirostis*) were categorized as critically endangered due to rapid population declines (Bird life International 2001). The proximate cause of decline was identified as high rates of mortality from renal failure (Gilbert *et al.* 2002) and renal failure was found to be due to the toxic effect of diclofenac residues in livestock carcasses (Oaks *et al.* 2004). Cunningham *et al*. (2003) reported that bursal lymphoid depletion and epithelialisation were found in 5 vultures and epithelial cysts were present in bursa of Fabricius of 4 vultures. However, they could not ascertain that the bursal regression was the only cause for immune suppression. Keeping all these reports in view, a comprehensive study was planned to investigate the toxicity, if any, due to diclofenac at sub-therapeutic dose either alone or under the influence of certain variables like modified diet (*i.e.*, high in protein, high in calcium and low in vitamin A), and induced immunosuppression in broilers.

Day-old sexed male broiler chicks (80) were divided into 8 groups of 10 chicks in each. All the birds were provided with feed and water *ad lib.* throughout the experiment. The groups were maintained as per the following schedule:

Group 1: Basal diet control (1–32 days)
Group 2: Basal diet for 32 days + diclofenac (0.8 mg/kg body weight i/m on day 24, 26, 28, 30 and 32)
Group 3: Basal diet for 32 days + cyclophosphamide (50 mg/kg body weight i/m once daily from day 20 to 23)
Group 4: High protein, high calcium, low vitamin A diet control (HPHC) (1–32 days)
Group 5: Basal diet + diclofenac + cyclophosphamide (as per the schedule)
Group 6: High protein, high calcium, low vitamin A diet + diclofenac (as per the schedule)
Group 7: High protein, high calcium, low vitamin A + cyclophosphamide (as per the schedule)
Group 8: High protein, high calcium, low vitamin A diet + diclofenac+ cyclophosphamide (as per the schedule)

The basal diet contained 37% protein and 1% calcium, whereas high protein high calcium low vitamin A (HPHC) diet contained 48% protein and 3% calcium. Both the diets were balanced energetically. Diclofenac was given after confirming immunosuppression in groups 5 and 8.

Blood samples were collected at the end of 34th day for determination of serum creatinine and prostaglandin E_2 (PGE_2; Elisa kit). Kidney samples were collected for antioxidant profile, diclofenac residue analysis, histopathology and electron microscopy. TBARS (Subramanian *et al*. 1988), catalase (Caliborne 1985), reduced glulathione (GSH) (Moron *et al*. 1979), SOD (Marklund and Marklund 1974) and diclofenac residues (Oaks *et al*. 2004) were estimated in kidney tissue at the end of 34th day.

The necropsy lesions of dead birds were recorded. The kidney samples were collected in 10% formal saline and processed for histopathology. Also kidney samples were collected in gluteraldehyde and processed for transmission electron microscopy (TEM).

The data were analyzed by one-way ANOVA using

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statistical package for social sciences (SPSS) version 10.

The serum creatinine concentration (mg/dl) in the basal diet control (group 1) was 0.547±0.021, which increased significantly (P<0.05) in all the remaining groups (Table 1). Serum creatinine was estimated as it forms the bio-marker for renal damage. The concentration of serum creatinine was significantly elevated in all the groups as compared to control group 1. Highest values were recorded in group 8 that was given diclofenac along with high protein diet and immunosuppression induced by cyclophosphamide. These results indicate compromised renal function.

The concentration of thiobarbituric acid reacting substances (TBARS; nM MDA/g protein) of kidney in the basal diet control (group 1) was 87.917±3.87, which was significantly (P < 0.05) increased in diclofenac-treated groups, while the concentration in groups 3 and 4 did not differ significantly from the basal diet control. The TBARS concentration in group 7 significantly (P<0.05) increased as compared to basal diet control. The reduced glutathione (GSH) concentration (mg/g protein) of kidney in the basal diet control (group 1) decreased significantly (P<0.05) in diclofenac treated groups, while the GSH concentration in groups 3, 4 and 7 did not differ significantly as compared to the basal diet control. The activity of SOD (units/g protein) of kidney significantly (P<0.05) increased in groups 2, 3, 5, 6, 7 and 8, whereas, the activity of group 4 (46.798 ± 3.052) did not differ significantly as compared to basal diet control. The activity of catalase M/min) of kidney significantly (P<0.05) increased in groups 2, 3, 5, 6, 7 and 8, whereas, the activity in group 4 did not differ significantly as compared to group 1 (Table 2).

Table 1. Results of renal biomarkers

Groups	Serum creatinine (mg/dl)	PGE_2 (pg/ml)	Diclofenac concentration* (mg/g tissue)
1. (Basal diet)	0.547 ±0.011^{A}	179.833 ±2.24^{C}	–
2. (Diclofenac (DCF))	0.713 ±0.019^{BC}	167.833 ±1.400^{C}	0.085 ±0.027
3. (Cyclophosphamide (CP))	0.687 ±0.013^{B}	221.667 ±9.718^{D}	–
4. (HPHC)	0.777 ±0.037^{BCD}	133.833 ±4.053^{B}	–
5. (DCF+CP)	0.822 ±0.588^{CD}	172.000 ±5.125^{C}	0.150 ±0.022
6. (DCF+HPHC)	0.811 ±0.556^{BCD}	60.414 ±7.509^{A}	0.046 ±0.014
7. (HPHC+CP)	0.756 ±0.033^{BC}	212.560 ±17.638^{D}	–
8. (DCF+HPHC+CP)	0.900 ±0.056^{D}	141.500 ±5.099^{B}	0.025 ±0.087

P=<0.05

HPHC-High proten high calcium low vitamin A diet; values are mean ± SE (n = 6);* mean ± SE (n = 4); one -way ANOVA (SPSS); means with different superscripts are significantly different (P<0.05).

The bio-markers of oxidative stress and anti-oxidant defenses revealed a significant increase in the activity of SOD and catalase and concentration of TBARS in kidney, while the concentration of GSH in kidney reduced significantly in the groups 2, 5, 6 and 8 that were treated with diclofenac either alone or under the influence of other variables in test. These results suggested an ongoing peroxidative stress and compromised antioxidant defense mechanisms. Hickey *et al.* (2001) reported that diclofenac has the ability to provoke massive oxidative stress *in vivo*. Vulture susceptibility to diclofenac results from a combination of an increased ROS, interference with uric acid transport and the duration of exposure (Naidoo and Swan 2009). Further, these findings can be substantiated from the histopathology of kidney that revealed degenerative changes in tubular epithelium. The histopathological sections of the kidneys from groups 2, 3, 4 and 7 revealed mild degenerative changes in tubular epithelium and inter-tubular haemorrhages (Fig.1). Groups 5, 6 and 8 revealed marked inter-tubular congestions (Fig.2) degenerative changes in tubular epithelium and disrupted tubular epithelium and architecture (Fig.3), whereas the other groups did not reveal the changes of any significance. The electron microscopy of PCT of group 2 showed vacuolation, condensation of chromatin, granular mitochondria without crystagalli inside, enlarged endoplasmic reticulum (Fig. 4). Group 5 showed chromatin condensation and rupture of mitochondria. Group 6 showed apoptotic nucleus, ruptured mitochondria. In group 8, PCT showed vacuolation, condensation of mitochondria, change in the shape of nucleus, mitochondrial size was reduced and there was nucleus fragmentation (Fig. 5). Semi-thin sections of kidney revealed extensive damage of PCT and mild changes in DCT. In group 2, PCT showed ruptured brush border, vacuolation in PCT and increased Bowman's space. Groups 5, 6 and 8 revealed extensive haemorrhages, increased podocytes and increased Bowman's space (Fig. 6). The HPHC diet induced nephritis and degenerative changes in various tissues of chicks (Chandra *et al.* 1984). Also, cyclophosphamide (immuno-suppressant) treatment induced segmental necrosis of lining epithelial cells of renal tubules (Kim *et al.* 2003). Diclofenac is a potent inhibitor of prostaglandin synthesis in mammals (Todd and Sorkin 1988) and inhibits prostaglandin synthetase and COX–2, both of which mediate production of prostaglandins E_2 and I_2 in smooth muscles of mammals (Vinals *et al.* 1997). In this study, there was a significant decrease in the concentration of PGE_2 in the groups that were given diclofenac in combination with HPHC diet. If the diclofenac increases or prolongs adrenergic opening of the renal portal valves, blood would be shunted away from the renal cortex. In addition, adrenergic vasoconstriction of the cortical vascular network would further decrease blood

Table 2. Results of oxidative stress

Groups	TBARS (nM/g protein)	GSH (mg/g protein)	SOD (units/g protein)	Catalase (µM/min)
1. (Basal diet)	87.917 ± 3.869^{AB}	16.922 ± 0.419^{DE}	44.365 ± 3.112^{A}	3.362 ± 0.244^{A}
2. (Diclofenac (DCF))	132.201 ± 9.857^{DE}	11.743 ± 0.536^{BC}	63.233 ± 1.542^{C}	5.507 ± 0.155^{C}
3. (Cyclophosphamide (CP))	108.434 ± 5.595^{BC}	14.0155 ± 0.392^{CD}	54.647 ± 5.106^{B}	4.332 ± 0.230^{B}
4. (HPHC)	84.469 ± 7.113^{A}	18.935 ± 1.181^{E}	46.798 ± 3.052^{A}	3.697 ± 0.318^{A}
5. (DCF + CP)	136.590 ± 5.811^{E}	7.792 ± 0.343^{A}	75.168 ± 2.152^{D}	5.500 ± 0.136^{D}
6. (DCF + HPHC)	134.951 ± 12.663^{DE}	10.838 ± 0.545^{B}	78.352 ± 1.694^{D}	5.952 ± 0.112^{D}
7. (HPHC + CP)	112.927 ± 7.514^{CD}	15.490 ± 0.698^{D}	56.163 ± 1.598^{B}	4.495 ± 0.220^{B}
8. (DCF + HPHC + CP)	130.235 ± 4.230^{CDE}	9.769 ± 0.847^{AB}	80.852 ± 1.743^{D}	5.927 ± 0.187^{D}

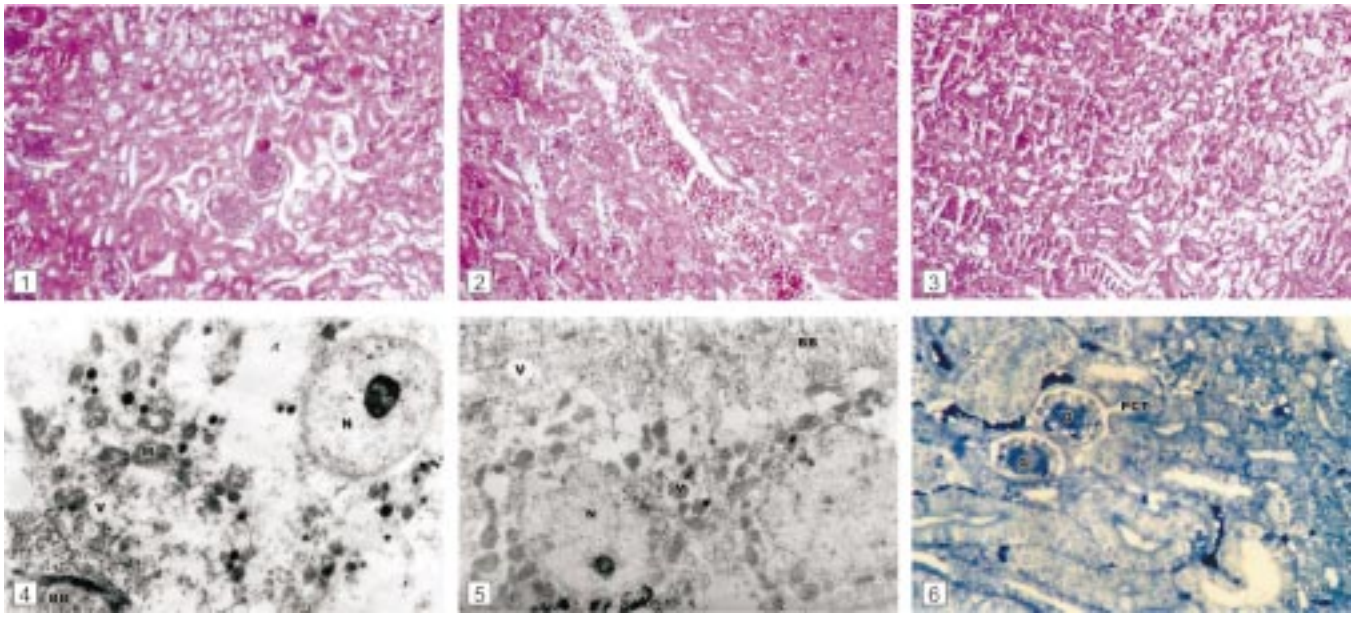
P=<0.05 HPHC-High proten high calcium low vitamin A diet; values are mean ± SE (n = 6); one -way ANOVA (SPSS); means with different superscripts are significantly different (P<0.05).

supply to the renal cortex. Diclofenac-induced ischaemia may induce necrosis of the proximal convoluted tubule (PCT).

The PGE_2 concentration significantly (P<0.05) decreased in groups 4, 6 and 8, while significantly (P<0.05) increased in groups 3 and 7. No change in PGE_2 concentration was observed in groups 2 and 5 as compared to group 1. The concentration of diclofenac in kidney of groups 2, 5, 6 and 8 did not reveal significant difference (Table 1).

Among the sacrificed birds, only the birds of groups 6 and 8 revealed mild congestion and enlarged lobes of kidneys. High dietary calcium and protein causes hyperuricaemia, which results in the occurrence of typical visceral gout. High calcium is said to be the primary cause of visceral urate deposition, while high protein diet is the secondary cause (Guo *et al*. 2005). In this study, visceral gout was prominent in the birds that were supplemented with the HPHC diet either alone or in combination with diclofenac. The birds that were treated with diclofenac alone did not reveal visceral gout. This may be attributed to the synergistic effects of diclofenac with high protein, high calcium diet that resulted in impaired renal function resulting in impaired uric acid excretion with subsequent deposition of urate crystals on several organs and organ parenchyma to cause visceral gout. Diclofenac affected the mitochondria resulting in compromised ATP synthesis either by hypoxia or cytotoxicity in the PCT that contributes to hyperuricaemia (Meteyer *et al*. 2005). This hypothesis confirms the findings of present study, which revealed elevated creatinine, and degenerative changes in PCT epithelium and apoptotic nuclei.

The mortality pattern in this study revealed the death of 4 birds out of 10 (40%) in group 6 (diclofenac + HPHC), followed by group 3 (cyclophosphamide; 20%), group 5



Figs 1–6. Photomicrograph of kidney showing: **1.** Mild degenerative changes in tubular epithelium H&E 100×; **2.** Marked inter-tubular congestion H&E 100×; **3.** Marked degenerative changes and tubular disruption H&E 100×; **4.** TEM of PCT showing condensation of chromatin and vacuolation (V) 8950×; **5.** TEM of PCT showing vacuolation (V), condensed mitochondria (M), changed shape of nucleus (N) 8950×; **6.** Semi-thin section of kidney showing increased Bowman's space, inter-tubular haemorrhages 400×.

(diclofenac + cyclophosphamide; 20%), group 8 (diclofenac + HPHC + cyclophosphamide; 20%) and group 2 (diclofenac; 10%) during the fifth week. Mortality was not recorded in the remaining groups. These results suggested that the mortality enhanced when diclofenac was under the influence of other variables. This is attributed to the synergistic toxic effect of different variables on the vital organs like kidney.

From our findings, it can be concluded that diclofenac has the toxic potential in poultry at sub-therapeutic doses and further the toxic effects are more pronounced under the influence of immunosuppressants and HPHC diet. These findings on diclofenac confirm the reports on vultures as far as the toxic effects are concerned, though the mortality in poultry was not very high at the dose tested, which may be due to the reason that both these species belong to two separate infraclasses (*Eoaves* and *Neoaves*, respectively). This is regarded as one of the deepest phylogenetic branches amongst living birds (Sibley *et al*. 1988).

SUMMARY

A comprehensive study was planned to investigate the toxicity, if any, due to diclofenae at sub-therapeutic dose either alone or under the influence of certain variables like modified diet (i.e. high in protein, high in calcium and low in vitamin A), and induced immuno-suppression in Broilers. It can be concluded that diclofenae has the toxic potential in poultry at sub-therapeutic doses and further the basic effects are more pronounced under the influence of immuno-suppressant and HPHC (high protein + high calcium) diet.

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