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## PATHOLOGY AND PROPOSED PATHOPHYSIOLOGY OF DICLOFENAC POISONING IN FREE-LIVING AND EXPERIMENTALLY EXPOSED ORIENTAL WHITE-BACKED VULTURES (*GYPSS BENGALENSIS*)

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**ABSTRACT:** Oriental white-backed vultures (*Gyps bengalensis*; OWBVs) died of renal failure when they ingested diclofenac, a nonsteroidal anti-inflammatory drug (NSAID), in tissues of domestic livestock. Acute necrosis of proximal convoluted tubules in these vultures was severe. Glomeruli, distal convoluted tubules, and collecting tubules were relatively spared in the vultures that had early lesions. In most vultures, however, lesions became extensive with large urate aggregates obscuring renal architecture. Inflammation was minimal. Extensive urate precipitation on the surface and within organ parenchyma (visceral gout) was consistently found in vultures with renal failure. Very little is known about the physiologic effect of NSAIDs in birds. Research in mammals has shown that diclofenac inhibits formation of prostaglandins. We propose that the mechanism by which diclofenac induces renal failure in the OWBV is through the inhibition of the modulating effect of prostaglandin on angiotensin II-mediated adrenergic stimulation. Renal portal valves open in response to adrenergic stimulation, redirecting portal blood to the caudal vena cava and bypassing the kidney. If diclofenac removes a modulating effect of prostaglandins on the renal portal valves, indiscriminate activation of these valves would redirect the primary nutrient blood supply away from the renal cortex. Resulting ischemic necrosis of the cortical proximal convoluted tubules would be consistent with our histologic findings in these OWBVs.

**Key words:** Diclofenac, *Gyps bengalensis*, nonsteroidal anti-inflammatory drugs, Oriental white-backed vulture, pharmaceutical residues, renal portal system, renal tubule necrosis, visceral gout.

### INTRODUCTION

Diclofenac, a nonsteroidal anti-inflammatory drug (NSAID), has caused sustained population declines in *Gyps* vultures of Pakistan, India, and Nepal. This is the first time a pharmaceutical compound has placed wild populations at risk of extinction (Oaks et al., 2004; Shultz et al., 2004; Green et al., 2004). This report describes the pathology of the Oriental white-backed vultures (*Gyps bengalensis*; OWBVs) from Pakistan that were naturally poisoned and experimentally exposed to diclofenac during a 2000–03 study (Gilbert et al., 2002, 2004; Oaks et al., 2004).

Peer-reviewed publications describing

pathology caused by NSAID use in birds are few. The pathophysiology of NSAID toxicity in birds has not been described and there are no published reports of diclofenac use in birds. Evidence of reversible clinical renal insufficiency with reduced creatinine clearance has been documented within 1 hr of NSAID administration in humans (Murray and Brater, 1993). Diclofenac-associated renal failure has been reported in humans with prolonged exposure (Murray and Brater, 1993) or with pre-existing renal disease (Davies and Anderson, 1997); these reports do not describe renal pathology. We propose a mechanism of action for diclo-

fenac toxicity in OWBVs in an attempt to explain the observed pathology and the extreme toxicity of this drug in OWBVs at doses that are safe and therapeutic for mammals.

#### MATERIALS AND METHODS

Tissues from 55 OWBVs poisoned by diclofenac and that died with visceral gout were examined microscopically. Thirty-nine of these OWBVs were found dead at roost sites in Punjab Province, Pakistan, and were suitable for histopathology and 16 OWBVs were captive and received a single oral dose of veterinary-grade diclofenac (0.25–2.5 mg/kg) or voluntarily ate 0.007–0.940 mg/kg of diclofenac as residue in a single meal of goat or buffalo tissue. Details of the field work and experimental exposure of OWBVs to diclofenac can be found in Oaks et al. (2004). Tissues collected from roost sites from 14 additional OWBVs that died of causes that did not involve visceral gout were examined microscopically as were tissues from three OWBVs exposed to diclofenac experimentally but that remained clinically normal until euthanasia between 1 and 4 wk postexposure. Kidney, liver, spleen, and lung were collected from all OWBVs and fixed in 10% neutral buffered formalin, trimmed, embedded in paraffin, sectioned at 5  $\mu$ m, and stained with hematoxylin and eosin by using standard methods. In addition to those tissues collected from all vultures, heart, trachea, esophagus, proventriculus, ventriculus, intestine, cloaca, pancreas, thyroid gland, parathyroid gland, adrenal gland, thymus, gonads, bone marrow, muscle, spinal cord, peripheral nerve, and skin from many but not all of the vultures were also examined microscopically.

#### RESULTS

Kidney lesions in the 55 OWBVs that died with visceral gout were severe in all cases. Kidneys with early lesions were uncommon but were critical in defining the primary renal pathology, which consisted of acute necrosis of proximal convoluted tubules of the renal cortex. Extent of epithelial cell necrosis could be seen as a continuum within the same proximal tubule. Hypereosinophilic shrunken cell remnants and luminal necrotic cellular debris were present adjacent to large, swollen epithelial cells with hypereosinophilic granular cytoplasm and very large nuclei with

prominent nucleoli (Fig. 1A). Proximal convoluted tubules were not affected uniformly throughout the section of kidney, but sections of kidney with more extensive renal pathology had universal necrosis of proximal convoluted tubules. There was no evidence of tubular epithelial regeneration or interstitial fibrosis. Renal changes accompanying acute proximal tubule necrosis included precipitation of delicate uric acid crystals (Fig. 1B) in tubules or vessels or random distribution of these crystals, breaching boundaries of interstitial space, tubule basement membranes, and vessel walls. Although the cells of the glomerular Bowman's capsule were sometimes prominent, glomeruli were generally spared. One well-preserved kidney had scattered large glomeruli deeper in the lobule (suggesting "mammalian type" glomeruli) with thickened membranous changes and eosinophilic material, consistent with fibrin (thrombi), in glomerular capillary loops (Fig. 1C). In kidneys with more advanced lesions, the lumina of distal convoluted tubules and collecting ducts were dilated and contained cellular debris and acellular eosinophilic material, consistent with protein. The epithelium of these distal tubules occasionally appeared flattened but viable (Fig. 1C).

More advanced renal lesions were associated with large amorphous, pale eosinophilic to lightly basophilic amorphous material mixed with necrotic debris and surrounded by a thin rim of inflammatory cells. The increase in the number, size, and distribution of these amorphous aggregates (gout tophi) was considered to reflect the progression of the disease. Larger coalescing tophi severely disrupted renal architecture and obscured tubular changes (Fig. 1D). Inflammatory cell response was minimal in all kidneys and when present was associated with gout tophi. The severe renal lesions described above resembled the pictures of OWBVs from India (Mishra et al., 2002, p. 285, fig. 3).

Urate deposits on the surface of visceral organs (visceral gout) resembled clouds of

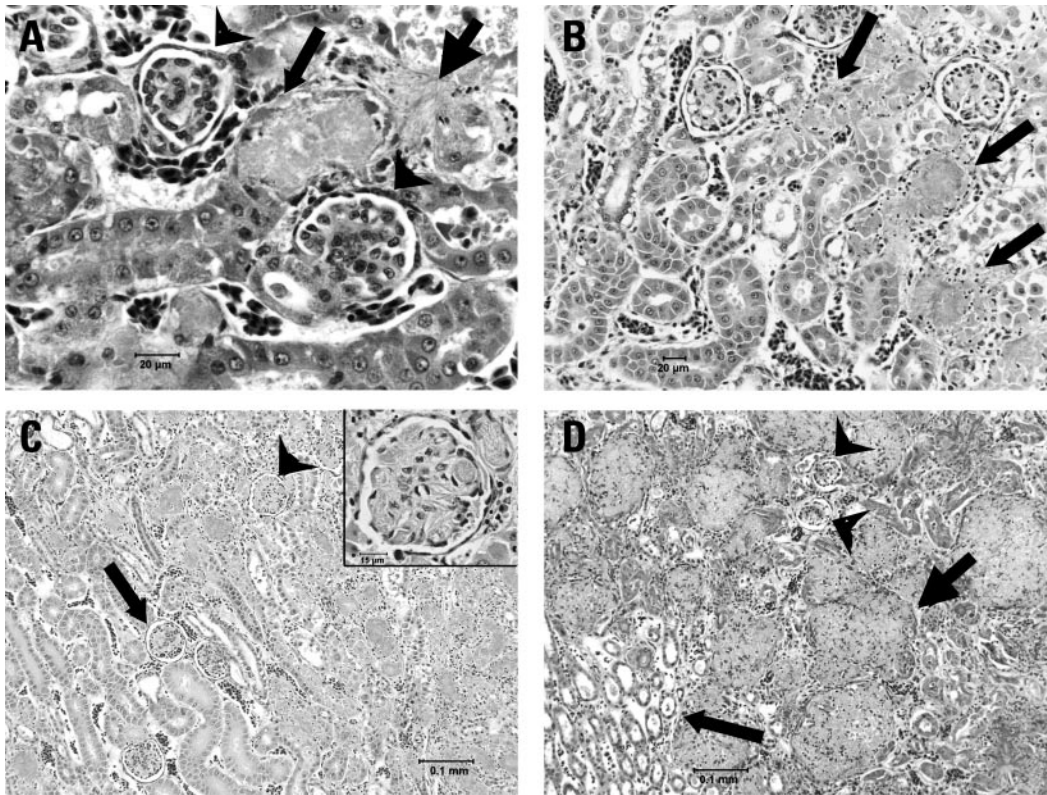


FIGURE 1. A: Kidney from a captive juvenile OWBV fed buffalo meat that contained  $6.4 \mu\text{g/g}$  diclofenac. Proximal convoluted tubule is lined by hyper-eosinophilic shrunken cell remnants (thin arrow) adjacent to cells that are swollen with prominent nuclei but are still viable. The regional glomeruli are unremarkable (arrowheads). Delicate lightly basophilic urate crystals cross tubular and vascular boundaries (thick arrow). There is no significant inflammation. B: Kidney from a captive juvenile OWBV given a direct oral dose of  $0.25 \text{ mg/kg}$  diclofenac. Lightly basophilic urate crystals are present in the lumen of proximal convoluted tubules lined with hyper-eosinophilic remnants of tubule epithelium with pyknotic nuclei (arrows). Regional glomeruli are unremarkable. C: Kidney from same vulture as Fig. 1B. Occasional glomeruli deeper in the renal lobule have membranous changes and eosinophilic material (arrow and inset), consistent with fibrin thrombi (arrowhead) in capillary loops. Regional proximal convoluted tubules are lined by acutely necrotic epithelial cells that contain pyknotic nuclei and are separating from each other and the basement membrane. Lightly basophilic silhouettes of urate crystals are present within tubules. Nuclei within viable swollen epithelial cells (lower left) are large with prominent nucleoli. D: Kidney from a wild juvenile OWBV found dead in Toawala, Punjab Province, Pakistan in 2002. Numerous large aggregates (gout tophi) of urate material with amorphous cell debris (thick arrow) obscure normal renal architecture and are very similar to pathology described in OWBV mortality in India (Mishra et al., 2002, p. 285, fig. 3). Inflammation is minimal. Distal convoluted tubule epithelium (thin arrow) is flattened but viable; the lumina contain scant eosinophilic proteinaceous material and cell debris. The glomeruli (arrowheads) are unremarkable.

gray-blue to faintly eosinophilic loose fibrillar material. Organ parenchyma often had randomly scattered, multifocal to coalescing crystalline arrays, consistent with silhouettes of uric acid crystals (Fig. 2). These crystals were associated with regional tissue necrosis but had minimal, if any, inflammation, suggesting that the crystals

precipitated in organ tissue very close to time of death. In addition to the kidney, uric acid crystals were seen most commonly in liver, spleen, lung, and heart, but were also noted in skin, adrenal gland, and parathyroid gland. Once a vulture reached the stage of saturated hyperuricemia that resulted in precipitation within as well as

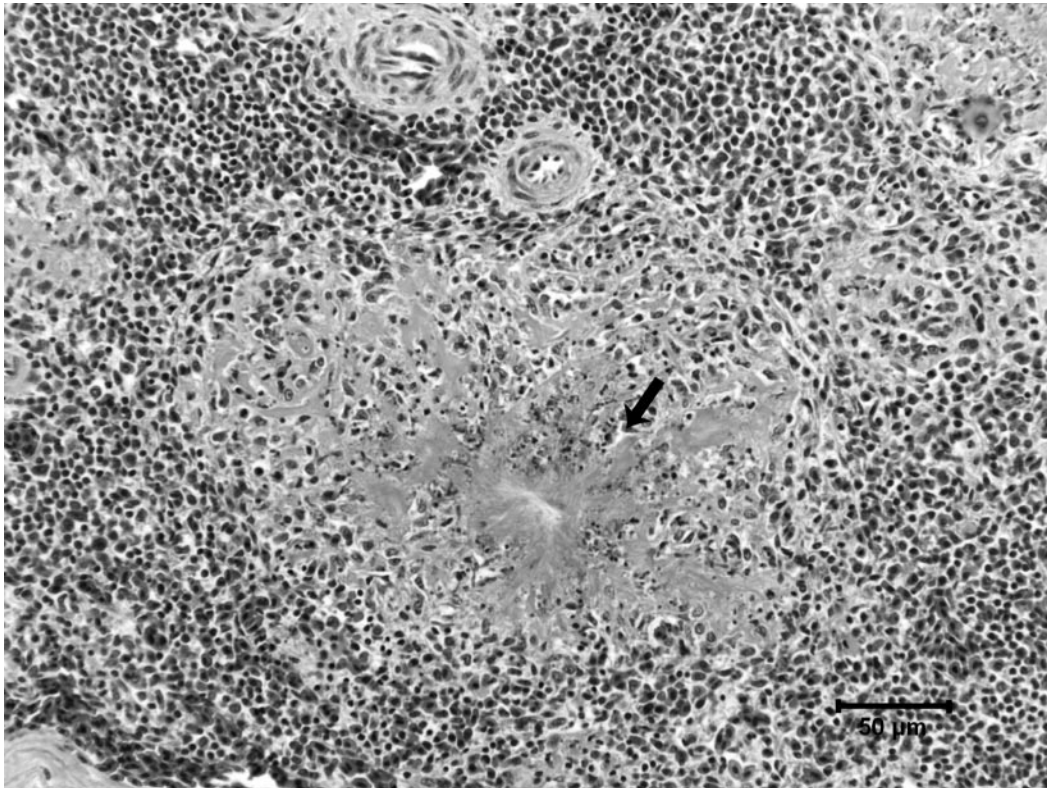


FIGURE 2. Spleen from same vulture as Fig. 1A. Urate crystals randomly deposited throughout the spleen parenchyma are associated with necrotic tingibile debris and pools of acellular eosinophilic material consistent with protein (arrow).

on visceral organs, urate deposition in the kidney was extensive and obscured renal architecture.

The renal pathology in the OWBVs dying from experimental diclofenac exposure was indistinguishable from the pathology in the kidneys of the free-living vultures that died with visceral gout. Kidneys from dead OWBVs collected from roost sites but that died from other causes and the three vultures that survived experimental exposure to diclofenac had no renal lesions.

Previously reported analytical results (Oaks et al., 2004) from kidneys of 25 of the wild birds with visceral gout and 15 experimentally exposed OWBVs with severe proximal convoluted tubule necrosis confirmed diclofenac residues between 0.051 and 0.643 ppm wet weight. No diclofenac was detected in the kidneys from

the 14 wild OWBVs that died without visceral gout or in the kidneys of the three experimentally exposed OWBVs that survived and were euthanized 8 days or 4 wk postexposure.

#### DISCUSSION

Kidney pathology was acute and severe in the OWBVs that died of diclofenac exposure. Although the extent of pathology varied among birds, none of the renal lesions in the poisoned vultures was considered truly chronic. The pathology in the wild birds also suggested a single initiating event versus multiple intermittent insults that would have produced varying degrees of lesion chronicity within a single vulture kidney. The acute tubular necrosis without evidence of repair or significant inflammation, and the adequate body fat stores in the dead birds, also suggested a short

duration of illness and rapid lesion progression to death. This was also consistent with the pathology subsequently seen in the experimental vultures that died over a range of days following experimental exposure. Figure 1A and B best illustrates the rapid progression from necrosis of renal tubules without urates to those that contain urate crystals, suggesting rapid precipitation of urates within the tubule lumen as the epithelium of the proximal convoluted tubules dies. This site of early uric acid precipitation is consistent with the role of the proximal convoluted tubules as the primary functional site of energy-dependent uric acid excretion in birds (Siller, 1981; Goldstein and Skadhauge, 2000).

Prior to the acceptance of diclofenac as the cause of mortality, renal disease in OWBVs in India was thought to be secondary to dehydration (Cunningham et al., 2003). Although dehydration can exacerbate the effect of NSAIDs in mammals (Hao et al., 2000), dehydration alone in birds has not been shown to cause the type of pathology seen in the OWBVs. Authors frequently cite Siller (1981) when listing dehydration as the cause of severe renal disease and death associated with extensive visceral gout. However, in his fascicle, Siller remarks that there is poor documentation of pathology, in particular, histopathology, in cases of birds with death due to water deprivation and dehydration. In a subsequent report of poultry deaths due to dehydration, Julian (1982) described progressive obstruction of the ureters which resulted in “ascending” renal disease in severe cases with the microscopic changes in the kidneys secondary to urate deposition in the ureters and distal ducts. Because renal pathology in the OWBVs began in the renal cortex rather than as an ascending progression of pathology beginning in the ureter and collecting ducts, the pathology was not consistent with dehydration. Acute necrosis of the proximal convoluted tubules in the OWBVs occurred in the absence of urate deposition supporting the

conclusion that the tubular epithelial necrosis was the cause, not the result of, urate deposition. The avicide 3-chloro-*p*-toluidine hydrochloride (DRC-1339 aka *Starlicide*) is known to cause acute renal tubule necrosis, visceral gout, and death in birds (Decino et al., 1966) but is not sold in Pakistan.

The selective necrosis of cortical proximal convoluted tubules and the relative sparing of the collecting tubules and glomeruli in birds poisoned by diclofenac suggest a mechanism of action that is either specific for the cortical region of the renal lobule or specific for the cells of the proximal convoluted tubule. The damaging effect of NSAIDs on mammalian kidneys is most commonly due to their effect on renal vasculature and blood supply (Murray and Brater, 1993). Prostaglandins E<sub>2</sub> and I<sub>2</sub> function as renal vasodilators in mammals and regulate renal blood flow supplied primarily through the afferent arterioles (Verlander, 1997). Diclofenac is a powerful inhibitor of cyclooxygenase-2 (COX-2) and prostaglandin synthetase (Vinals et al., 1997), both of which are involved in prostaglandin E<sub>2</sub> production. Blood flow to the avian kidney is very different from blood flow in kidneys of mammals. The renal portal system, via the afferent renal portal vein (Fig. 3), is the primary nutrient blood source for the avian renal cortex and does not supply the renal medulla or medullary cone (Braun, 1993; Goldstein and Skadhauge, 2000; Smith et al., 2000). The primary blood supply to the avian glomeruli and distal convoluted tubules is the central artery located between the intralobular (central) vein and the cortex. Blood supplied by the central artery leaves the kidney via the efferent intralobular vein at the center of the renal lobule. Because blood to the proximal convoluted tubules is supplied by the renal portal system, the effect of diclofenac on this system warrants further discussion.

There is species variability in the anatomy of the renal portal system (Johnson, 1979) and the specific anatomy of these

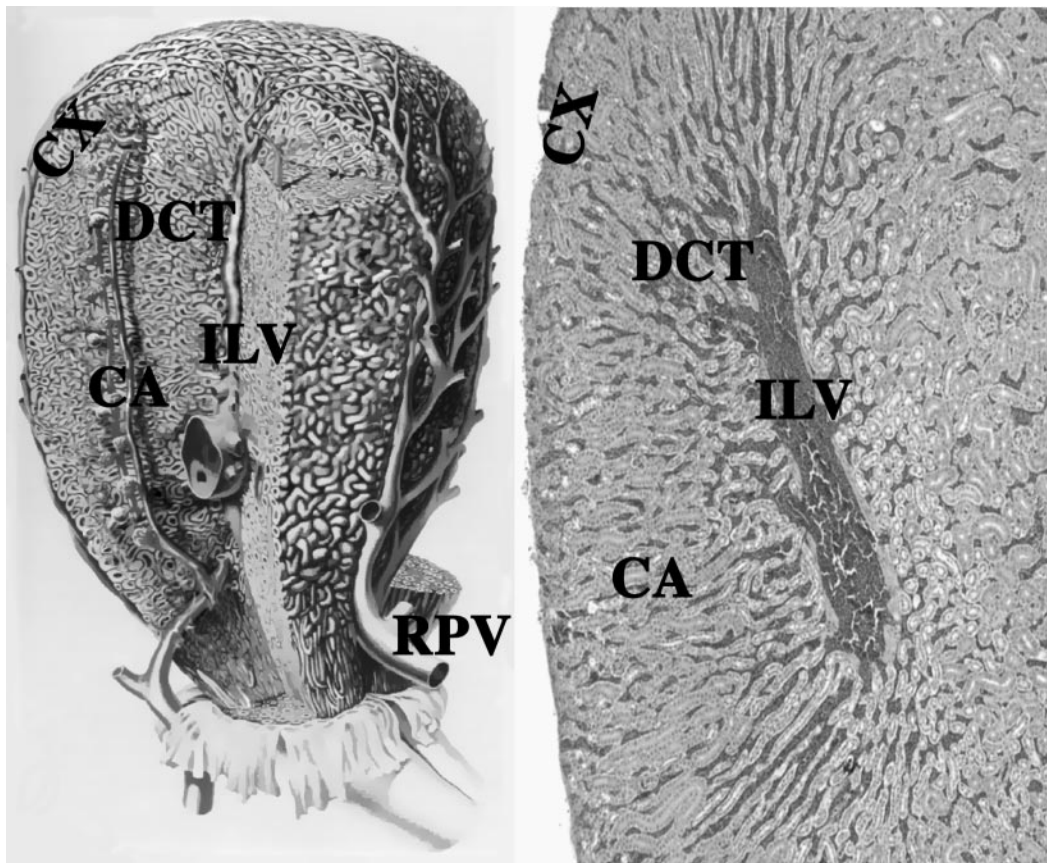


FIGURE 3. Anatomic model of the avian kidney (modified from Siller, 1981, p. 191, fig. 1) compared with a histologic section of a normal OWBV kidney. CX, renal cortex with proximal convoluted tubules; CA, central artery on the model and path of the central artery on the histologic section although the artery itself is not evident; ILV, efferent intralobular vein; DCT, distal convoluted tubules that surround the intralobular vein; RPV, afferent renal portal vein, not represented in the histologic image.

structures in the OWBV is unknown. As a generalization, the renal portal vein in the external iliac vein controls the major portion of the blood flow through the renal portal system (Braun, 1993). Blood flow to the renal cortex is under multiple levels of adrenergic control. The smooth muscle sphincters of the renal portal valve are highly innervated by adrenergic and cholinergic nerves. Adrenergic stimulation of the renal portal valve shunts blood away from the renal portal vein into the common iliac veins which merge to form the caudal vena cava (Fig. 4), thus bypassing the kidney. In addition, adrenergic stimulation increases tension and reduces blood flowing through the interlobular afferent

veins and its branching vascular network and peritubular sinuses that course around and through the cortex (Johnson, 1979; Goldstein and Skadhauge, 2000; Smith et al., 2000).

The presence of a functional angiotensin system has been documented in birds (Goldstein and Skadhauge, 2000), although details of this system are less understood in birds than in mammals. Angiotensin II is continuously produced maintaining blood pressure by mediating the activation of adrenergic nerve fibers with subsequent vasoconstriction. Angiotensin II-converting enzyme in bird kidneys exceeds that in the lung (Henderson et al., 1993). The angiotensin II-mediated vaso-

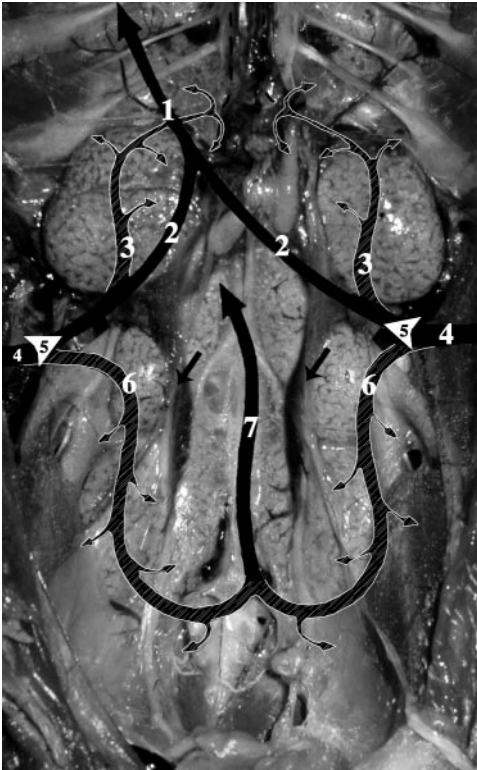


FIGURE 4. Experimental dosed OWBV died 58 hr after receiving 2.5 mg/kg diclofenac orally. Kidneys are swollen with prominent tubular pattern. Normal ureters are empty, translucent, and difficult to visualize (black arrows). The superimposed diagram of the renal portal blood flow is modified from Johnson (1979, p. 215, Fig. 4.21C). The veins outlined in white represent the renal portal system and the black veins direct blood flow away from the kidney. The renal portal valve (5, white arrowheads) is near the branching of the external iliac vein (4) which forms the common iliac vein (2), the cranial renal portal vein (3), and the caudal renal portal vein (6). The caudal renal portal veins merge to form the caudal mesenteric vein (7). When the renal portal valve is open in response to adrenergic stimulation, the renal portal blood preferentially flows into the common iliac veins, bypassing the kidney, and enters the general venous circulation as the common iliac veins merge to form the caudal vena cava (1).

constriction in mammals is modulated by compensatory release of prostaglandins  $E_2$  and  $I_2$  in the kidney, which promote vasodilation (Murray and Brater, 1993; Verlander, 1997). Diclofenac is a potent inhibitor of prostaglandin synthesis in mammals (Todd and Sorkin, 1988) and inhibits pro-

taglandin 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). The mechanism of action of diclofenac in birds is unknown, but the roles of prostaglandins  $E_2$  and  $I_2$  and COX-2 in the smooth muscle control of the renal portal valve of OWBVs merits further investigation.

If diclofenac increased or prolonged 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 supply to the renal cortex. Although very low doses of diclofenac are tolerated by OWBVs (Oaks et al., 2004), a threshold of diclofenac-induced ischemia may exist beyond which necrosis of proximal convoluted tubules would ensue. Once sufficient proximal convoluted tubules died, a “point of no return” might be reached from which the vultures would not recover. The dying proximal convoluted tubules would no longer contribute to the regional prostaglandin production with subsequent exacerbation of cortical vasoconstriction initiated by diclofenac. Proximal tubules are also the primary site of uric acid excretion and reabsorption of ultrafiltrate (Henderson et al., 1993). Necrosis of proximal convoluted tubules would compromise uric acid excretion, leading to a rapid elevation of uric acid concentration in blood. Once the saturation point in the blood is reached, uric acid would rapidly precipitate as crystals on organ surfaces and within organ parenchyma resulting in death.

The kidney is the primary site of diclofenac excretion in mammals (Davies and Anderson, 1997). If renal disease is present, excretion of diclofenac conjugates would be reduced. As diclofenac conjugates accumulate in the blood, they readily hydrolyze to re-form the active parent compound prolonging the toxic effects of diclofenac contributing to a fatal cycle (Murray and Brater, 1993). Almost no work has addressed the absorption, distri-



bution, metabolism, and excretion of NSAIDs in avian species.

The literature discussed above supports adrenergic control of renal portal valves and describes the renal portal system as the primary blood supply for the renal cortex. These are fundamental assumptions on which our hypothesis for the mechanism of action of diclofenac in OWBVs is constructed. Results from an experiment with six anesthetized domestic turkeys (*Meleagris gallopavo*) treated with epinephrine, however, questioned the effect of epinephrine on the patency of the renal portal valve as well as the importance of the renal portal system in the blood flow to the kidney (Palmore and Ackerman, 1985).

Another possible explanation for the preferential necrosis of the proximal convoluted tubules caused by diclofenac may be related to the high metabolic activity of these cells which would make them more sensitive to hypoxia than cells in the distal or collecting tubules that are less metabolically active (Brown, 1985). The direct effect of the parent drug on mitochondria resulting in compromised ATP synthesis and the cytotoxic effect of metabolites also need to be considered (Bort et al., 1999). Because uric acid excretion occurs at the proximal convoluted tubules and is an energy-dependent process (Siller, 1981; Goldstein and Skadhauge, 2000), decreased ATP, either by hypoxia or direct cytotoxicity, would contribute to hyperuricemia.

Diclofenac may affect the kidney of various genera of birds differentially. Unlike *Gyps* sp., population declines in the Egyptian and king vultures (*Neophron percnopterus* and *Sarcogyps calvus*, respectively) and other scavenging birds have not been documented on the Indian subcontinent (Prakash, 1999; Gilbert, unpubl. data). Although the Egyptian and king vultures differ from *Gyps* vultures in their feeding habits and food preferences (Houston, 1985), the possibility of differential physiologic response to diclofenac may also play

a role in their survival. Regulatory paths of nutrient blood supply to the renal cortex differ between species (Goldstein and Skadhauge, 2000) and the sensitivity of the renal portal valves to diclofenac may also vary between species.

Reports document variable response of different avian species to NSAIDs. Renal tubular necrosis, visceral gout, and mortality occurred in king eiders and spectacled eiders treated with ketoprofen but the mortality in king eiders was greater at lower doses than in spectacled eiders (Mulcahy et al., 2003). A study of flunixin in quail (Klein et al., 1994) reported glomerular pathology, which might suggest a change in regulation of blood flow from the central artery to the glomeruli in quail, rather than an effect on the renal portal system. Results of a 36-hr clinical study by Baert and De Backer (2003) compared the plasma clearance rates of three NSAIDs in five bird species. The authors concluded that, because the difference in protein binding alone was so great between species, the pharmacokinetics of each NSAID should be independently assessed for each target avian species. More research using clinically relevant treatment regimens, assessment of physiologic parameters, and histopathology are needed to more clearly define the toxic affects of NSAIDs in birds.

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