Diclofenac in Gyps vultures: A molecular mechanism of toxicity

Vinny Naidoo

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Department of Paraclinical Sciences
Faculty of Veterinary Science
University of Pretoria
South Africa

Diclofenac in Gyps vultures: A molecular mechanism of toxicity

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VINASAN NAIDOO

Department of Paraclinical Sciences
Section of Pharmacology and Toxicology
Faculty of Veterinary Science
University of Pretoria

2007

Promoter: Professor GE Swan
Dean: Faculty of Veterinary Science
University of Pretoria

Declaration

The experimental work described in this thesis was conducted in the department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Section of Pharmacology and Toxicology under the supervision of Prof GE Swan.

These studies are the result of my own investigations, except where the imputs of others are acknowledged. This thesis has not been submitted to another university for consideration.

I, Dr Vinasan Naidoo, declare the above statement to be correct

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Dr V Naidoo

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Prof. GE Swan

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Abstract

Diclofenac in Gyps vultures: A molecular mechanism of toxicity

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Vinasan Naidoo

Promoter: Prof GE Swan

Department: Paraclinical Sciences

Degree: PhD

Over the last decade, three species of Gyps vultures on the Asian subcontinent have declined dramatically in population numbers, some as much as 97 to 99%. Although the initial cause was believed to be infectious, it was later shown to be due to an inadvertent exposure to diclofenac via the food chain. In order to protect the remaining wild vultures, diclofenac needed to be removed from the food chain. Unfortunately the Indian government was reluctant to ban diclofenac until an alternate veterinary non-steroidal anti-inflammatory drug (NSAID) that was both safe in vultures and effective in cattle could be identified. Although meloxicam was tentatively identified as this drug, toxicity testing still needed to be undertaken.

Using a previously validated model, two studies were undertaken to determine the acute toxic effect of diclofenac in vulture as well as to ascertain if the drug had the potential to accumulate. In the first study, meloxicam in formulation was shown to be safe as a single oral dose up to 2mg/kg in African White Backed-Vultures (*Gyps africanus*). To further demonstrate the safety of food borne meloxicam, vultures were exposed to meat rich in meloxicam residues, with once again no signs of toxicity being evident. In the second study the drugs ability to accumulate was evaluated pharmacokinetically in Cape Griffon Vultures (*Gyps corprotheres*). From this study meloxicam was shown to have a very short half-life of elimination, making it unlikely that the drug could be a cumulative toxin. This was subsequently confirmed clinically by the absence of toxicity in birds receiving repeated doses of meloxicam.

Although meloxicam was shown to be adequately safe, the safety of other veterinary NSAIDs still required elucidation. While further testing in vultures would have been possible, the small population size of the various vulture species made this unethical. Therefore a surrogate species needed to be identified. With the domestic chicken (*Gallus domesticus*) being commonly available, attempts were made to validate the chicken as a model. Although the dosed chickens did show similar toxicity patterns from clinical pathology to histopathology, a major problem was their higher tolerance making it impossible to use them as a surrogate. It was, however, concluded that the domestic chicken may be used in mechanistic studies in an attempt to establish an *in vitro* model.

From the mechanistic studies both diclofenac and meloxicam were directly toxic to chicken and vulture renal tubular epithelial cells following 48h of incubation. It was later shown that this toxicity was associated with an increased production of reactive oxygen species (ROS), which could be temporarily ameliorated by pre-incubation with uric acid due to its anti-oxidant activity. When cultures were incubated with either drug for only two hours, meloxicam showed no toxicity in contrast to the cellular toxicity present for diclofenac. In both cases no increase in ROS production was evident. In addition diclofenac influenced the excretion of uric acid by interfering with p-amino-hippuric acid channels. The effect on uric acid excretion persisted after the removal of the diclofenac. It was therefore concluded that vulture susceptibility to diclofenac results from a combination of an increase in cellular ROS, a depletion of intracellular uric acid concentration and most importantly the drug’s long half-life in the vulture. Unfortunately the importance of the drug’s half-life in the toxicodynamics makes it unlikely that *in vitro* testing will be possible.

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Abbreviation

ALT:	Alanine transferase
AST:	Aspartate aminotransferase
AUC:	Area under curve/Extent of absorption
AWBV:	African White-backed vultures
***C*:**	Plasma concentration at time *t*
Ca^{2+}:	Calcium
CINODS:	Cyclo-oxygenase inhibiting Nitric Oxide Donors
CK:	Creatine kinase
COX:	Cyclo-oxygenase
DAD:	Diode array detector module
DF:	Diclofenac
DMEM:	Debulco's modified Eagles's essential medium with L-glutamine
DMSO:	Di-methyl sulphoxide
FCS:	Foetal calf serum
$F_{relative}$:	Relative bioavailability
H:	Heterophil
Hb:	Hemoglobin concentration
HBSS:	Hanks balanced salt solution
Hct:	Hematocrit
HPLC:	High performance liquid chromatography
i.m.:	Intramuscular
IUCN:	International Union of the Conservation of Nature
K^{+}:	Potassium
K_a:	Absorption constant,
K_e:	Elimination constant
Ln:	Natural logarithmic
LOD:	Limit of detection
LOQ:	Limit of quantitation
LOX:	Lipo-oxygenase
MCHC:	Mean corpuscular hemoglobin concentration

MCV:	Mean corpuscular volume
MLE	Maximum level of exposure
MLX:	Meloxicam
MMP:	Mitochondria membrane permeability
MRP:	Multiple Resistance Protein
MTT:	3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
Na^+:	Sodium
NE:	Norepinephrine
NO:	Nitric oxide
NRF:	National Research Foundation
NSAID:	Non-steroidal anti-inflammatory drug
OAT:	Organic anion transporters
PAH:	p-Amino-hippuric acid
PBS:	Phosphate buffered saline
PCV:	Packed cell volume
PG:	Prostaglandins
PK:	Pharmacokinetics
PSS:	Physiological saline solution
RBC:	Total erythrocyte counts
REST:	The Rare and Endangered Species Trust
ROS:	Reactive oxygen species
RSPB:	The Royal Society for the Protection of Birds
RTE:	Renal tubular epithelial
$T_{1/2\alpha}$:	Absorption half life
$T_{1/2\beta}$:	Elimination half life
Tmax:	Time to maximum concentration
U:UA:	Urea: uric acid ratio
UA:	Uric acid
UPBRC:	University of Pretoria Biomedical Research Centre
URAT1:	Uric Acid Transporter 1
V_d/F:	Apparent volume of distribution
WBC:	Total leukocyte count
ZSL:	Zoological Society of London

CHAPTER 1: Introduction

1.1 The Vulture Crisis

At least three species of vulture, endemic to South Asia, are in grave danger of extinction across the Indian subcontinent[54,106,122]. Populations of Oriental white-backed vulture (*Gyps bengalensis*), long-billed vulture (*G. indicus*) and slender-billed vulture (*G. tenuirostris*) have declined by more than 97% in India and Pakistan with the annual rates of decline, appearing to be on the increase[54,106,122]. Due to these declines, all three species were listed by IUCN, The World Conservation Union, as Critically Endangered[15].

Research by Oaks *et al.*, 2004, first indicated diclofenac, a non-steroidal anti-inflammatory drug (NSAID), as the only cause of the observed rapid population decline across the Indian subcontinent in 2003[100]. This indicated that the catastrophe was purely secondary, following exposure to diclofenac residues in the food source. This problem has since been reproduced under controlled experimental conditions in captive Asian white-backed vultures[100].

From the current literature published for mammalian species, diclofenac is a typical NSAID which works by suppressing inflammation, pain and fever[112]. Diclofenac, as with other first generation NSAIDs, inhibits the activity of both the cyclo-oxygenase-1 and cyclo-oxygenase-2 enzymes to produce their beneficial effects. This mechanism is unfortunately also related to their toxicophoric effects such as gastric ulceration, renal toxicity and impaired liver function[20]. In addition to the typical use of the NSAIDs diclofenac is an important component in the control of gout in people. This effect is directly opposite to that seen in vultures i.e. the drug treats gout in people and yet is the major cause of gout in vultures[112].

Although the use of diclofenac has been conclusively shown to be the cause of the vulture population decline, the mechanism of toxicity has not been adequately explained. At present the only consistent change present at all post mortem examinations was severe

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Picture of a golden vulture [89]; Picture of vulture with outspread wing[4]

diffuse visceral gout, which tends to suggest the kidneys or its supportive circulatory system are the target for drug toxicity[90,100].

More importantly the decline in the vulture numbers needs to be stemmed. At present the Royal Society for the protection of birds, has set up numerous breeding colonies in India to achieve this goal. Unfortunately with vulture pairs producing only one egg a year, these breeding centres will never re-populate the species. This has therefore made it very important to prevent further losses in the current vulture population. A major obstacle to achieving this has been the Indian Governments refusal to ban the sale and manufacture of diclofenac, due to its importance in a holy animal. They, however, made the proviso that their stance may be re-considered if an alternate vulture-safe NSAID were to be described.

1.2 Hypotheses

i. Meloxicam as an alternative non-steroidal anti-inflammatory drug is safe in vultures.
ii. Diclofenac toxicity results from the inhibition of uric acid transporters in the renal tubular epithelial cells.

1.3 Objectives

i. To establish the safety of meloxicam as an alternate NSAIDS for use in cattle.
ii. To establish baseline clinical pathology of *Gyps africanus*.
iii. To establish the domestic chicken as a surrogate model for toxicity testing and comparisons.
iv. To establish the mechanism of diclofenac toxicity.

CHAPTER 2: Literature Review

2.1 Vultures: Twenty-first century outcasts

They are not cute, cuddly and will never inspire that "ooh-factor" we reserve for predators such as cheetahs, lion, leopards and eagles. Neither will they inspire that grudging respect that has been earned by predators such as crocodiles, sharks and tigers. None the less, vultures are still an integral component of the environment in which they form the apex of the detrivorous food chain[37]. In addition to the clearing of carcasses, current research tends to suggest that these birds may even play a role in minimising the spread of diseases such as anthrax and possibly even keeping rabies at bay by indirectly decreasing the mingling of predators at feeding sites[63,94,127].

As a species, vultures are characterised by their large size, large feather-less heads, curved beaks and are renowned for being some of the highest fliers ever[143]. They are further differentiated from other raptors by being predominantly carrion eaters in that they will only resort to predation in times of extreme food shortages[126]. The vulture family, which is rather large, can be further divided into the old-world and the new-world vultures (Figure 2-1). The former, also known as the Griffon Vultures being the descendants of the original vultures, while the latter, such as the condors, having evolved from the stork, which is an oddity in nature, as two genetically unique species under different selection pressure ended up following the same convergent evolutionary pathway[143].

Although not the most beautiful species to observe, vultures have managed to capture the imagination of people. In early Africa dating back to the seat of modern civilization, the vulture held a special place in the hearts of the Egyptians (Figure 2-2)[1,4,89,123]. With the birds being the highest flier, they were close to the sun god Ra, which was something the Egyptian people strived for. Additionally the vulture, specifically *Gyps fulvus* (Griffon vulture) was the symbol of the upper kingdom of Egypt and thus the mark of the God-king Pharaoh of the day.

Picture of a golden vulture [89]; Picture of vulture with outspread wing[4]

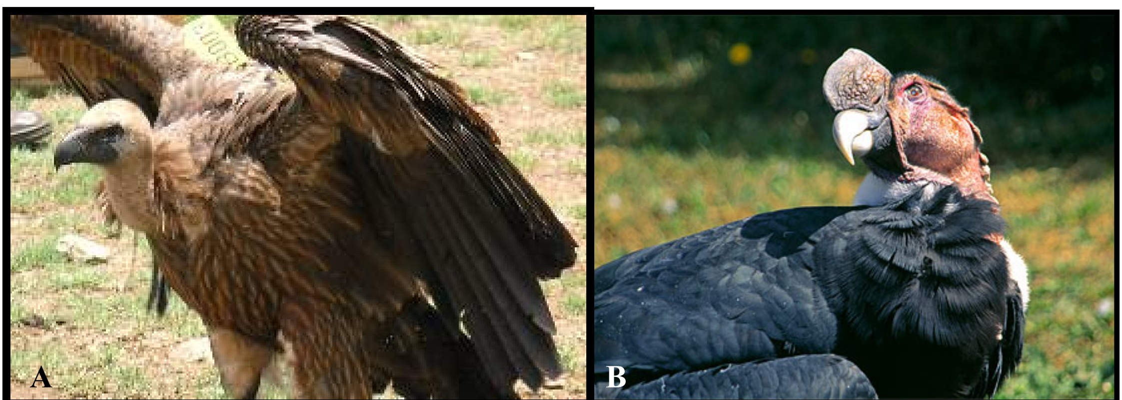

Figure 2-1: The two major branches of the vulture family

A-An old world griffon vulture (*Gyps africanus*) (Picure taken at Licthenberg),

B- A new world vulture (*Vultur gryphus*)(Picture from National Geographic)[69]

The high esteem, at which vultures were held, was not just restricted to western civilisation, but extended to the east (Figure 2-2). In the Ramayana, an important Hindu scripture, the vulture king played an important role in the fight of good against evil[3]. In its religious verse, the Ramayana describes how the vulture king tries to protect Sita from the evil Ravena, only to be beaten by the embodiment of ultimate evil.

Alas this past sphere of privilege endowed onto the humble vulture has largely fallen away in these modern times and has probably resulted from its common association with death. This misconception is still perpetuated by the popular press, television and Hollywood cinematography, where it's still common to see vultures circling characters near death, marking gloomy spots such as cemeteries or being associated with witch-craft (Figure 2-3).

Figure 2-2: The importance of the vulture to early civilisations

- **A- A gold vulture representing the upper kingdom on the death mask of the child-king Tutankhamun (Picture obtained from the Science Museum)[2],**
- **B- The evil Ravana defeating the vulture king following the battle described in the Ramayana (Picture obtained from wikipedia)[3]**

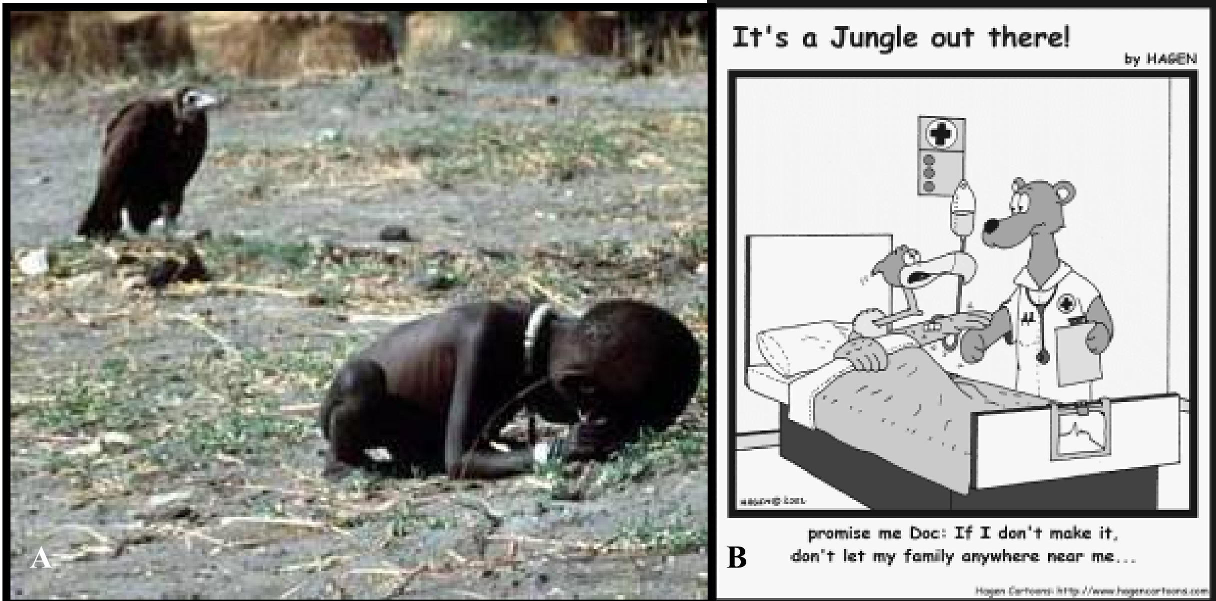

Figure 2-3: Vulture being shown as evil creatures, interested in only death, by popular press and cartoons

- **A- Pulitzer prized picture taken by photojournalist Kevin Carter, showing a vulture hovering over a weak girl taken in Sudan (1993). Not shown in the picture was that the weak girl walked away after the picture was taken[9]**
- **B- A vulture cartoon portraying the birds as nothing more than cannibals.[55]**

Even Africa's population, which initially (through their Egyptian Ancestry) held the vulture in high regard, has largely turned its back on them. Nowadays more value is placed on the dead vulture than those soaring free. As a result of the practice of the muti trade which is also referred to as African witchcraft, muti-men and other death dealers place much value of certain appendages of these birds (Figure 2-4)[140]. The eyes are meant to be a doorway to the future for clairvoyance, the adage of the lucky rabbit foot has been extended and the now "lucky vulture foot" should help you claim some of the lottery millions or alternatively one's courage may be boosted by covering oneself in a layer of vulture fat[33,66,76,140]. More recently is has also been suggested that eating the brain of a vulture will also produce a state of clairvoyance (K Wolter, 2007, Pers comm.)

Figure 2-4: Vulture heads and feet being sold for use as muti at a Malay market (Picture from Science magazine)[76]

In addition to the muti trade, vultures are constantly facing other challenges in their environment such as exposure to poisons placed out to kill troublesome predators and even deliberate persecution by farmers who have the mistaken view that vultures are linked to losses of lambs and calves[137]. Then there is also their continual exposure to dwindling ecosystems that stem from the expansion of human dwellings or manmade hazards such as power lines and electrical pylons[6]. And lets not forget the influence of diseases that have stemmed from manmade manipulations of the environment[88,121].

2.2 A crash in the Indian Vulture Population

2.2.1 *No longer the world's most prominent birds*

Even though vulture numbers, around the world, have been steadily declining due to persecution and other problems as described above, it was nothing compared to the ultimate species devastation that occurred on the Indian Subcontinent. What was once described as the most prominent bird species in the world (Figure 2-5), no longer held this title[48]. In less than fifteen years the Asian White-backed vulture (*Gyps bengalensis)* has passed from being the top high-flier in the world to the most endangered species, following an astounding and catastrophic collapse in population numbers in excess of 95% (Figure 2-6), throughout the Indian subcontinent (India, Pakistan and Nepal)[101,106,118]. In addition to the white-backed vulture, other *Gyps species,* the Long-Billed (*G. indicus*) and Slender-billed (*G. tenuirostris*) vultures have also suffered similar losses and are listed by the International Union of the Conservation of Nature (IUCN) as critically endangered[15]. More recently the Egyptian vulture (*Neophron percnopterus*) and red-headed vulture (*Sarcogyps calvus*) have also been shown to be declining[34].

The first occurrence of mortalities in the white-backed vulture was reported in the Keoladeo National Park during the 1996-97 nesting season by Prakash *et. al.* (1999)[105]. In this study it was reported that the birds were dropping dead from their roosts or were found dead on their perches, in branches, or in their nests. A catastrophe that was not just limited to the adults but also fledglings, as they too were being found dead near their nests. In addition to the abnormal deaths, specific clinical signs were described. The afflicted vultures were observed to be sick for a variable amount of time that could extend up to 32 days. Typically, the vultures appeared drowsy with a limp, dangling neck (described as depression)(Figure 2-6). After appearing to wake up by raising its head, the bird would once again succumb to this depressed state. The end result was similar, as in all cases the birds died. In addition, reproductive failures consisting of either failure to lay eggs, failure to hatch, an increased mortality in the hatchlings or combinations thereof were observed[105].

Figure 2-5: Pictures taken in the early 1980's showing the prominence of the Oriental White-back vulture in India (Courtesy of the RSPB)

Following post-mortem examination of the dead birds, the necropsies were all characterised by the presence of whitish crystals, assumed to be urates, on the liver, heart, kidney and spleen (Figure 2-7: A)[109,110]. On histopathology the most striking lesions were present in the kidneys, and included degenerative changes in the urinary tubules and the presence of deep eosinophilic epithelial cells, many with absent nuclei (Figure 2-7). Urate tophi, evident as radiating eosinophilic masses and mononuclear cell infiltration, particularly lymphocytes and monocytes, were present around the glomeruli.

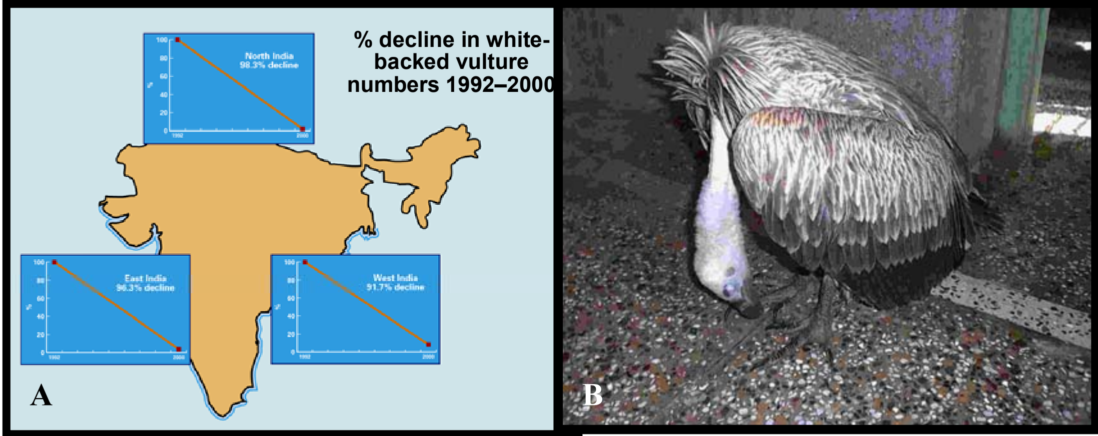

Figure 2-6: Illustration of the catastrophic decline in the vulture numbers (Courtesy of the RSPB)

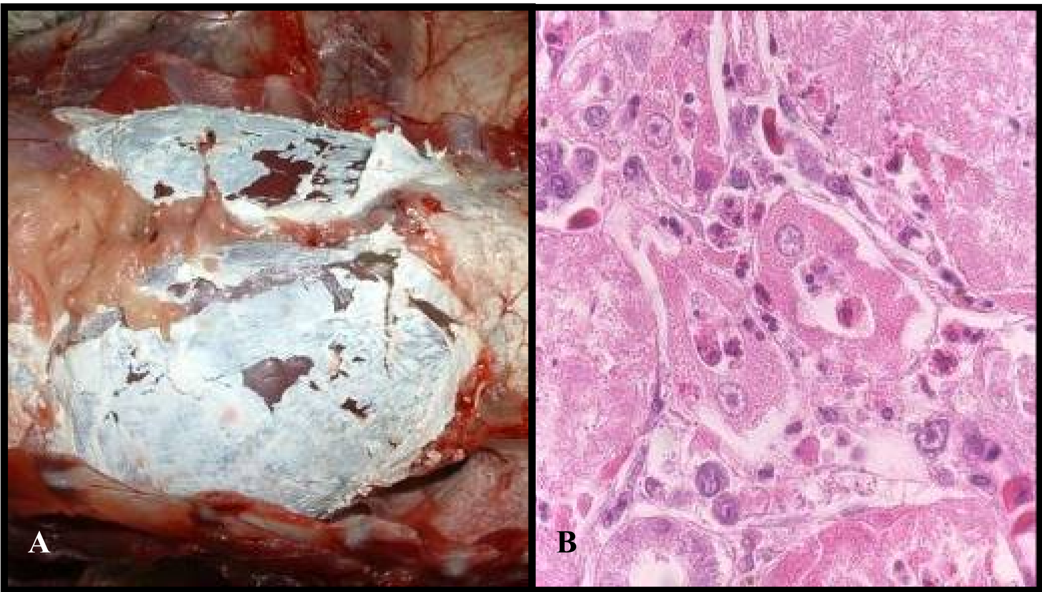

Figure 2-7: Typical necropsy and histopathological lesions seen in the poisoned birds

- **A- Diffuse pasty white material covering the abdominal viscera (Pictures courtesy of Prof L Oaks**
- **B- Histopathological lesion showing renal tubular damage (Pictures courtesy of Prof L Oaks)**

Although the end result of mortalities and reproductive failures in combination with gout-like signs were seen in the affected colony, the causal factor was as that stage unknown. Researchers from the Zoological Society of London (ZSL) and the Royal Society for the Protection of Birds (RSPB) were convinced that it had to be an infectious agent, although extensive microbiological investigation yielded no infectious aetiology[100,101]. The study did, however, reveal a confounder to an infectious condition, as it was limited to the white-backed vultures and not found in the two other species of vultures in Keoladeo National Park viz. the Egyptian vulture (*Neophron percnopterus*) and the king vulture (*Sarcogyps calvus*) at that stage.

It was also proposed that poisonings could be the cause, as suspected pesticide poisoning was reported in crows in the vicinity of Bharatpur and Agra[118-121]. But once again the screening of carcasses for the commonly used pesticides in the area failed to demonstrate sufficiently high levels to explain the reported deaths[100]. Additionally none of the currently available pesticides have been known to cause the specific clinical signs reported in the vultures.

From the failure of other tests to conclusively demonstrate the presence of a toxin or infectious agent(s), other mortality factors such as food shortages and human persecution

were also proposed at the time[118-121]. Food shortages were easily ruled out by the presence of abundant fat reserves in the abdominal cavities, in at least two of the necropsies, as well as the presence of abundant unconsumed carcases in the areas where birds were found dead. Although the abundance of a food source was important in the epidemiology of the condition, the first break-through in the aetiology, only became evident at some point later, in studies undertaken by Oaks *et al.*, 2004[100].

In a controlled study, Oaks and co-workers were able to demonstrate that diclofenac, a rather innocuous veterinary non-steroidal anti-inflammatory drug (NSAID), was the cause of the vulture mortalities right down to the characteristic histopathological lesions. In addition, trace concentrations of diclofenac were detected in twenty-three vultures that died in the field with signs of visceral gout. Oaks *et al.*, 2004, were also able to demonstrate that residues of diclofenac in carcasses, originating from treated animals, were sufficiently high (0.005 to 1.0 mg/kg) to be lethal to a vulture consuming a single large meat meal. Since then it has also been statistically demonstrated that as little as 1% of the total carcasses available for vulture food needed to contain diclofenac to cause the devastation seen[54].

2.2.2 *Why were the birds exposed to diclofenac?*

This is probably the most important question that has been raised since the species crashed. To answer this question we should first consider both the veterinary and religious policies in India and Pakistan as diclofenac residues in cattle carcasses was purely from veterinary treatment prior to their death.

As in other countries it was common practice in both India and Pakistan to treat sick animals with NSAIDs for either their palliative analgesic or anti-inflammatory effect[18]. The difference on the Indian subcontinent was in their selection of diclofenac as their NSAID of choice, in the early 1990's[141]. The drug had the advantage of not only being widely available, manufactured by approximately forty different pharmaceutical companies, but was also very cheap, more so in India due to a governmental subsidy. The net effect was that a large number of animals at any given point were being treated with diclofenac. Although this would explain how cattle got exposed to diclofenac, it does not take into consideration the religious implication in both Pakistan and India.

One of the predominant religions in India is Hinduism, a diverse religion based on the principle of polytheistic monotheism. In Hinduism the cow is seen as a holy animal as she is the protector of the holy text, the four books of the Vedas[56,57,130]. More practically they are symbolic of life as they provide life sustaining milk and are the source of subsequent generations of life i.e. they produce calves. Even though the milk produced by cattle is important in human nutrition, it is valued more for its religious properties as it can be converted into ghee (clarified butter) which symbolises purity and features in all Hindu religious ceremonies. Cattle are also a source of dung, which has become an important fuel source as well as a disinfectant when burned. In the rural farming communities oxen are important beasts of burden that are vital for the short planting season that follow the monsoon rains. It has been observed that farmers, who failed to secure sufficient oxen to plough their farms, have had to give up farming and move to the cities. Overall, the cow brings more to a poor Hindu family than just a source of meat. With the living animal being of such high importance, it is not surprising that cattle are never slaughtered by Hindu families i.e. animals usually die naturally. Sick animals are always given every opportunity to recover. In most cases this involves the use of diclofenac. As such it is not surprising that a large number of dead carcasses in India have diclofenac residues as these animals will have been treated until either recovery or death, especially with euthanasia not being an option.

In contrast to India, Islam is the predominant religion in Pakistan[72]. Here cattle are kept for meat production, in accordance with the teachings of the Prophet Mohammed[75,130]. Although the prophet imparted a number of important messages to his people, one of the more important teachings was a prohibition on the slaughter of sick animals for human consumption in a practice that is still considered relevant these days, in order to prevent the transmission of illnesses to people[128]. As such, sick animals are never consumed in Pakistan. However, as in India, these sick animals do get treated with NSAIDs, once again diclofenac, in the hope that they would recover. Unfortunately this has also resulted in animals being treated until they succumbed to their illness.

From the religious and treatment practices in both India and Pakistan it is easy to see how diclofenac ended up in the cattle carcasses, but it fails to explain how these carcasses ended

up in the vulture food chain. One peculiarity in both countries was in their management of the carcasses of dead cattle. Unlike other countries, where carcasses are destroyed by incineration, rendering or burial, both countries placed the carcasses out for the feeding of vultures in the practice known as vulture restaurants[111,130]. This practice of vulture restaurants in combination with normal veterinary practice was unfortunately the catalyst for the vulture population devastation, as it inadvertently introduced the toxin to the vultures.

2.3 Impact of a Declining Vulture Population

With the steady decline of vultures, an apex predator, major changes are becoming evident in the communities that are very dependant on them[86].

2.3.1 *Aesthetic value*

To understand the importance of the vulture to the Indian ecosystem, one has to consider the practice of the vulture restaurants as mentioned above. In addition to disposing of the carcasses of animal dying after an illness, vultures in India were also responsible for the clearing of carcasses left out from the leather industry as well as offal from abattoirs[86,131]. As a result vultures had become India's proficient carrion disposers. White-back vultures are so efficient that they could strip an entire carcass in twenty minutes, due to their large numbers at the carcass.

With the decline in the vulture population, the ability of these birds to dispose of carcasses has been tremendously reduced, with the result that is now common to find carcasses that have never been fed upon, especially in areas where vultures were once a common site[86]. This has resulted in carcasses rotting in the environment creating an aesthetically unpleasing smell in village rubbish dumps. Additionally these dump sites have attracted unwanted pests such as rats which are transmitters of human diseases.

With the decrease in the vulture population it has been speculated that this could result in a concurrent increase in the incidence of anthrax, as the spread of *Bacillus anthraxis* spores is directly linked to the occurrence of predators[94]. Generally predators are involved with

the dispersal of the spores either mechanically due to the contamination of their hair, feathers or legs with blood; from clinical and subclinical infections; or via faecal excretion of undigested spores[62]. Even though vultures are mechanical vectors of anthrax, it has been shown that the anthrax bacillus is efficiently broken down by the vulture stomach, most likely due to the low pH, thereby limiting total environmental contamination[62]. With the decline in vulture numbers, a natural buffer that would normally decrease the total yield of spores has been removed, thereby increasing the potential for the spread of anthrax.

2.3.2 *Importance to the Parsi Community*

The Parsi community are members of the zoroastrinism community, which migrated to India from Persia and settled in small communities in the cities of Mumbai, Delhi, Lucknow and Ahmadabad.[86] Unlike Hinduism and Islam, the predominant religions on the Indian subcontinent, the Zooparsis believe in the invisible god. In their religion emphasis is placed on the purity of earth, water, air and fire (the basic elements) which have to be preserved. In keeping with the purity of nature, they believe that the bodies of their dead have the potential to contaminate the environment making burial and cremation an unacceptable method of disposal.

To allow for the disposal of their dead, bodies are exposed to the sun in burial towers (towers of silence) where solar radiation slowly incinerates the body[86]. With the large number of vultures in the India, they inadvertently became involved in the disposal ceremony which became known as sky burials. In fact vultures were simply a tool in the disposal of carcasses and not of any spiritual value. With the slow disappearance of the vultures, sky burials in India have become largely ineffective, making it necessary for the Parsi community to find other suitable burial methods.

At present large solar reflectors are used to concentrate solar radiation on bodies. With temperatures reaching up to 120°C the Parsi community claims that the body may be completely incinerated in three days. Unfortunately this may be an exaggeration as a recent newspaper report from India, by a Parsi widow, states that the solar reflectors are completely ineffective with the result that bodies slowly rot over a few months[117].

2.3.3 *Increase in the dog population*

Wild dogs have always been a problem in India. At the beginning of the 1990's it was estimated that nearly 18 million feral dogs were present throughout the country[86]. These dogs, like the vultures, derived their food from the large number of carcasses left out in the field. Although the dogs were always present in large numbers, vultures were still the apex predator in the country and with the average vultures consuming 0.5kg of meat (the equivalent of the total weekly feed intake of an average sized dog) no real competition existed between the species. With the rapid decline in vulture numbers over a fifteen year period, the dog population has been steadily increasing with the current population estimated to be near 29 million in 2004.

The increase in the number of dogs has created its own set of problems. With these dogs being completely feral they have no qualms about attacking people. Local statistics indicate that 2.06 bites occur annually for every 1000 people in the country.[86] Although the cost of treating bites wounds in India is a major expense, the real cost of the increase in the dog population could be an increase in the incidence of rabies[86,127].

2.3.4 *Loss of income to the bone collectors*

In India bone collection for processing as fertilizer, following the cleaning of carcasses by the vultures, had been an important form of income in rural areas due to the large population and poverty[86,130].The decrease in the vulture population has decreased the ability of these people to collect the bone from skeletons, limiting their source of income.

2.3.5 *Air travel*

Not all of India views the decrease in the vulture population as being undesirable[121]. The large vulture population in the 1980 & 90's had become a hazard to air traffic. These large birds had the potential and at time did damage engines and windshields of planes taking off and landing at airports. With the decline in the vulture numbers, air flights in India have become much safer.

2.4 NSAIDs: An Overview

Non Steroidal Anti-inflammatory Drugs (NSAIDs) represent some of the oldest medicines with a recorded history of use by people[42,139]. Although their use was initially restricted to herbal remedies the use of the NSAIDs has developed into a multi-billion dollar modern pharmaceutical industry, in which a large number of chemically pure compounds have been synthesised. Of these aspirin (acetylsalicylic acid), first chemically produced in large quantities by the Bayer Pharmaceutical Company, represents the typical example and most frequently used NSAID[139].

2.4.1 *Chemistry*

Although the NSAIDs may all achieve a similar therapeutic goal, they actually represent a fairly diverse group of chemical compounds that fall into the following categories[18,23,112]:

- The salicylates: are modifications of the highly irritant 2-hydroxybenzoic acid (salicylic acid). Of this group aspirin is the most recognised NSAIDs in the world. Other drugs in the class include olsalazine and sulfasalazine.

- Para-aminophenol derivatives (coal tar derivatives) are metabolites of phenacetin e.g. paracetamol (acetaminophen).

- Acetic acid derivatives: Includes the aryl and heteroaryl acetic acid derivatives e.g. tolmetin, ketorolac, etodolac, indomethacin, sulindac, and etodolac.

- The fenamates: The fenamates are a family of NSAIDs that are derivatives of N-phenylanthranilic acid and includes mefenamic, meclofenamic, and flufenamic acids.

- Phenylacetic acid derivative: Diclofenac is a phenylacetic acid derivative that was developed specifically as an anti-inflammatory agent.

- Propionic acid derivatives (profens): The arylpropionic acids are characterized by the general structure Ar-CH(CH_3)-COOH. The proprionic acid derivatives are one of the largest veterinary classes and include ibuprofen, ketoprofen and carprofen.

- Enolic acids (oxicams): The oxicam derivatives are enolic acids characterized by the 4- hydroxybenzothiazine heterocycle e.g. piroxicam and meloxicam.

- Phenylpyrazolone derivatives: This class of agents is characterized by the 1-aryl-3,5- pyrazolidinedione structure. This group of drugs includes phenylbutazone, oxyphenbutazone, antipyrine, aminopyrine, and dipyrone.

- Coxibs: are diaryl-5-membered heterocycles and are characterized by their COX II selective activity e.g. Celecoxib, rofecoxib, valdicoxib and firocoxib.

- Nimesulide: Nimesulide is a sulfonanilide compound available that demonstrates COX-II selectivity similar to celecoxib in whole blood assays

2.4.2 *Mechanism of Action*

As the name implies, the NSAIDs is characterised by an anti-inflammatory effect in the absence of a steroidal ring in the molecule. Although widely used over the last few hundred years, the exact mechanism of the class still remains largely speculative. At present the mechanisms describing the anti-inflammatory effects of these drugs are divided into the cyclo-oxygenase (COX) mediated and non-COX mediator mechanisms:

2.4.2.1 COX mediated effects

The majority of current literature suggests that the modulation of the COX enzymes viz. COX I to COX III as being the site at which these drugs function[17,18,49,92,138]. With COX being an important enzyme in the conversion of arachidonic acid into the prostaglandins (PGf2ά, PGE_2, and PGD_2), the prostacyclines (PGI_2 and PGX) and the thromboxanes (TXA_2 and TXB_2)(Figure 2-8), inhibition of this enzyme does partially explain the benefits of the class.

Figure 2-8: Metabolic pathways of the arachidonic acid cascade. NSAIDs: Non-steroidal anti-inflammator drugs; EETs: epoxyeicosatrienoiac acid; HETEs: hydroxyeicosatetraenoaic acids; PG: prostaglandin; TX: thromboxane[96]

- **COX I:**[23,49,92,107,138] Is present in nearly all parts of the body. The enzyme is responsible, for the maintenance of normal homeostasis e.g. haemostasis in all tissues, vascular tone and muscle contractility. They are a constitutive part in many tissues around the body and are necessary for the normal physiological functioning of the body. The COX I enzyme is, however, also inducible and may therefore

contribute to the inflammatory cascade, as seen with the COX II enzyme (see below).

- **COX II :**[23,49,92,107,139] Is very similar to the COX I enzyme in structure. Once formed, they play an important role in the pathology of inflammation. The inducible form of this enzyme is the most important and in certain sections of the spinal cord may also be a key mediator of pain and is also involved in the control of wound healing. In certain regions of the body such as the kidneys they are, however, still constitutive and play an important function in the maintenance of vascular tone.

- **COX III**:[28,142] This subtype of the enzyme (also referred to as the COX 1b/COX 1v enzyme), at present, has predominantly been isolated in the brain of dogs and appears to be involved in the transmission of painful stimuli from the area at which pain is felt to the central nervous system where pain impulses can be interpreted. Although the exact purpose of the enzyme is unknown, some authors suggest that COX III plays a role in inflammation while others suggest that the enzyme is not of any clinical significance[59,74].

For its potential involvement in inflammation, two alternate theories have been put forward to explain the mechanism by which COX III and the NSAIDs interact. The first theory postulates that the NSAIDs such as paracetamol stimulate COX III thereby enhancing the production of 15deoxy$\Delta^{12-14}PGJ_2$ which is a potent natural anti-inflammatory substance[28]. Up-regulation of the latter enzyme may be one of the reasons why the NSAIDs result in remission in people being treated with the NSAIDs in a pulse manner. An alternate body of literature suggests that COX III is an isomer of COXI and therefore a normal mediator of inflammation and pain. In this theory, COX III inhibition is suggested as the mechanism behind spinal analgesia[23].

2.4.2.2 Non-COX mediated Effects

In addition to the COX-mediated actions discussed above, the NSAIDs are also able to produce their beneficial effect by acting on alternate mechanisms. Although distinct for the COX enzyme, these mechanisms work in conjunction with the COX mediated effects of the drug:

- **Lipo-oxygenase (LOX) inhibition**: In addition to the COX pathway a second pathway known as the LOX pathway is present within cells (Figure 2-8)[96]. While the COX enzyme promotes the formation of the prostaglandins and prostacyclins, the LOX ezyme promotes the formation of the leukotrienes from arachidonic acid. In addition to being potent smooth muscle contractors, the leukotrienes are also important mediators of inflammation[32]. Leukotriene B4, in particular, has been associated with the recruitment of leukocytes to areas of inflammation, promoting the release of lysosomal enzymes by neutrophils, as well as enhancing overall plasma leakage[32]. Certain of the NSAIDs, known as dual inhibitors, have been shown to attenuate both the LOX and COX pathways thereby enhancing their overall anti-inflammatory activity[79]. In veterinary medicine, tepoxalin is the most recognised dual inhibitor[30].

- **Leukocyte Attenuation**: In addition to inhibiting the formation of the inflammatory mediators, the NSAIDs have the ability to modulate the functionality and activation of neutrophils and thus the inflammatory cascade directly at the cellular level[5,18]. Other beneficial effects include a decrease in the generation of superoxide ions, decreased release of lysosomal enzymes, the inhibition of lymphocytes activity and modulation of monocyte functionality[18,43,136].

- **Nitro oxide inhibition**: It has been suggested that the NSAIDs function by inhibiting the formation of nitric oxide (NO). In one study, using models of joint inflammation, exposure of cells to aspirin, ketoprofen or ibuprofen reduced the overall culture NO content and subsequently protected the culture from NO induced cellular apoptosis[67]. This ability of the NSAIDs to affect NO may been explained by a receptor coupled mechanism, as NO up-regulates both COX enzymes during times of inflammation thereby enhancing the formation of the pro-

inflammatory mediators i.e. by inhibiting COX the NSAID automatically modulate NO functionality[115,116]. Not all authors are, however, in support of this theory as it has been suggested the ketoprofen mediates its effect by stimulating serotonin receptors without influencing NO[38].

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- **Antineoplastic activity**: Certain of the NSAIDS also possess potent antineoplastic activity in both people (celocoxib, aspirin and sulindac) and dogs (piroxicam) which appears to result indirectly through the inhibition of the COX enzymes[17,27,47,51]. With the COX enzyme being inhibited, arachidonic acid is able to accumulate as the phospholipase A_2 enzyme remains unaffected. This subsequently, by a yet undescribed mechanism, stimulates the conversion of sphingomyelin to ceramide a potent apoptotic agent[27]. It has also been suggested that the non-COX mediated mechanism may be mediated by the downregulation by of proto-oncogenes, C-myc and the transcription factors PPARδ, NK-κB, PAR-4 and Bcl-2[64].

2.4.3 *Pharmacological Activity*

In general this class of compounds is characterised by the following pharmacological effects[17,18,49]:

- **Anti-inflammatory action**: The prostaglandins (PG) are important inflammatory mediators. Their effects include vasodilatation, increased vessel permeability and chemotaxis of inflammatory cells into the injured region. By decreasing the PG concentrations in the tissues, NSAIDs cause a corresponding decrease in the inflammatory response. In addition to the inhibitory effect on the production of PG, certain members of the class have the ability to interfere with the functioning and degranulation of neutrophils (e.g. aspirin). The NSAIDs also differ in their anti-inflammatory ability with paracetamol (acetoaminophen), which is completely devoid of anti-inflammatory activity most likely as a result of a difference in its specificity for the inducible COX II enzymes at the site of inflammation.

- **Analgesia**: At present the NSAIDs are widely used in the management of pain. Although their analgesic mechanism is poorly understood, it is believed that the

NSAIDs function by decreasing the formation of prostaglandins. During incidents of injury, there is generally an indirect increase in the PG concentrations in injured tissues. They are believed to be the stimulus for peripheral sensitisation (they lower the threshold of the peripheral nociceptors to mechanical and chemical stimuli) which is perceived by the higher nerve centres as pain. Their analgesic effect is purported to be due to a decrease in the accumulation of the mediators of pain at the site of injury.

- **Anti-pyrexia**: Certain infectious and inflammatory conditions cause an increase in the body core temperature i.e. pyrexia. This results from the induction of the COX II enzyme in the hypothalamic thermostat and the subsequent increase in PGE_2 concentrations. The increasing PG concentrations raise the hypothalamic thermostat set-point with a resultant pyretic reaction. The NSAIDs are able the reset the set-point to basal levels by inhibiting the COX II enzyme, thereby allowing for an alleviation of the pyretic reaction.

- **Uricosuric Effect:** The NSAIDs are important agents for the management of gout, an important condition in people characterised by the accumulation of uric acid in the blood and tissues. Certain NSAIDs decrease the accumulation of uric acid in the body by inhibiting selected uric acid transporters (Organic Anionic Transporters or SLC22a transporters), thereby promoting the net excretion of uric acid from the body (For more detail, see 2.5.2.2).

- **Other beneficial effects**: Although less frequently used, other beneficial effects include their ability to attenuate the inflammatory cascade during endotoxaemia, decreasing the coagulatory activity of platelets via thromboxane inhibition, anti-neoplastic effect and lastly decreased epidermal cellular division in cases of seborrhoea sicca.

2.4.4 *Adverse Drug Reactions*

Due to their beneficial anti-inflammatory and analgesic effect, the NSAIDS have become some of the most widely used drugs in both animals and people. Unfortunately their chronic use has been associated with the occurrence of severe gastric ulceration. In an attempt to limit this side effect various different subclasses of NSAIDs have been developed, resulting in a more modern classification of the NSAIDs based on their ability to inhibit the two main subclasses of the COX enzymes (COX selectivity is dependant on the species of use)[30,32,61,131]. The older generation of NSAIDs became known as non-selective inhibitors as they inhibited both the COX I & II enzymes e.g. aspirin, ibuprofen and naproxen. More selective agents known as COX II selective agents were soon discovered and were characterised by a lower incidence of gastric ulceration e.g. diclofenac, carprofen and meloxicam. The COX II specific drugs soon followed and were defined by their ability to inhibit only the COX II enzyme and were characterised by a negligible incidence of gastric ulceration e.g. cerecoxib, verocoxib and valdicoxib. The most recent addition to the class are the dual COX and lipo-oxygenase (LOX) inhibitors which inhibit the two major pathways involved in the metabolism of arachidonic acid e.g. tapoxalin. A fifth subgroup known as the COX inhibiting Nitric Oxide Donors (CINODS) is currently being investigated for their ability to reduce the incidence of gastric ulcerations.

As with their mechanism of action, the NSAIDs may also be characterised by specific side effects they induce:

- **COX I**:[23,40,96] *Gastric ulceration*: PGE_2 and PGI_2 regulate gastric acid secretion. They also regulate the secretion of the protective gastric mucus barrier, and promote normal gastric circulation. Inhibition of the COX I enzyme, results in a loss of the fine control of gastric acid production, leading to self-injury and ulcerations (Figure 2-9). *Renal perfusion*: As with other systems, the prostaglandins maintain renal haemodynamics as well as modulating the vasoconstrictor effects of endogenous mediators such as vasopressin, angiotensin II and adrenaline. The inhibition of PGE_2 thereby promotes renal ischaemia and ultimately results in renal papillary necrosis (For more detailed discussion see 2.5.2.1).

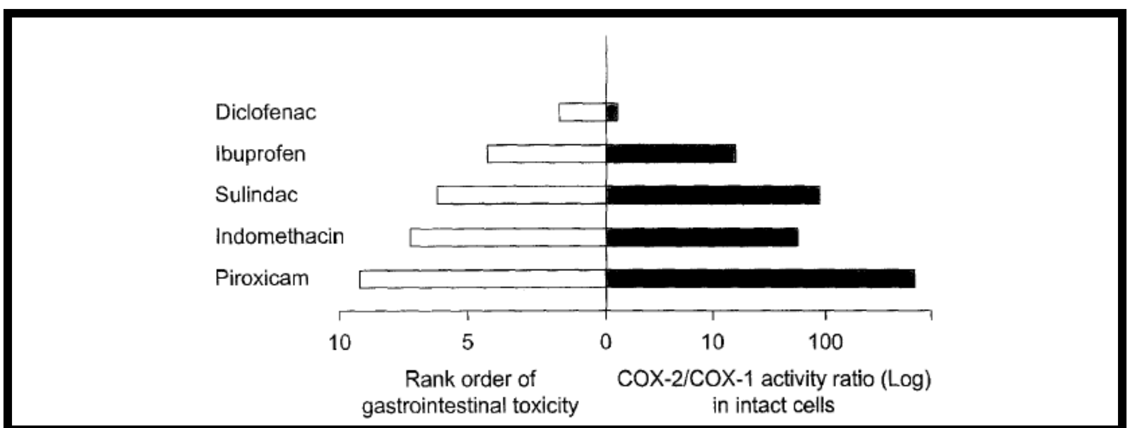


Figure 2-9: Comparison of gastric damage and COX selectivity of various NSAIDs used in people[96].

- **COX II:** Certain homeostatic mechanisms are maintained by the constitutive COX II enzymes, which are of specific importance in the kidneys. With their inhibition, renal perfusion may be affected as discussed above [20,71]. It has also been shown that the preferential inhibition of the COX II enzyme by the newer Coxib subgroup of drugs has the potential to induce COX I related side effects. Their selective inhibition of the COX II enzyme, results in a compensatory increase in the level of the COX I enzyme system, a subsequent increase in TXA_2 production and hypercoagulability of the blood[13]. In people this translates to cerebral and cardiac thromboembolic derived ischaemic necrosis.

In addition to the COX mediated adverse reactions discussed above, the NSAIDs may also be cellular toxins:

- **Toxicity as a result of biotransformation**: In veterinary medicine cats are highly susceptible to the metabolites produced during the metabolism of NSAIDs such as paracetamol. As a species, cats are deficient in the synthetic phase enzyme glucuronyl transferase which is important for the degradation of reactive oxygen species that are produced following phase I cytochrome P450 metabolism[17,18]. Metabolic toxicity is also not unique to veterinary medicine and has been reported for paracetamol and diclofenac in people (For a more detailed discussion on diclofenac see 2.5.2.3)[112].

- **Direct cytotoxicity**: The pyrazolone derivatives are probably the most potent cytotoxic NSAIDs available for use, of which phenylbutazone is regarded as highly toxic in people due to its ability to induce aplastic anaemia characterised by peripheral pancytopenia and bone marrow hypoplasia[23]. In addition to inducing blood dyscrasias in horses, phenylbutazone is directly irritant to the gastrointestinal mucosal membrane, inducing erosions along the entire gastro-intestinal tract and is also believed to be the cause of right dorsal colitis[68].

2.4.5 *Duration of Effect*

As a class the NSAIDs are characterised by different rates of metabolism[17,18,30]. Although this is important in the functioning of most drugs, the duration of action of the NSAIDs are more dependent on the pharmacodynamic half-life of the drug. It has been shown that the prolonged duration of effect of the NSAIDs is related, in part, to the high degree of protein binding of the drug. Under conditions characterised by inflammation, protein seepage occurs into the site of inflammation[18,30]. As a result albumin bound diclofenac seeps into the inflamed tissue and serves as a reservoir of the drug at the site of injury. Certain of the NSAIDs, such as aspirin, are also known to produce a long term effect by inhibiting the COX enzyme irreversibly. This has the advantage that a single exposure to the drug will result in a long term effect.

2.4.6 *Non-steroidal anti-inflammatory drugs in birds*

Although birds don’t respond to pain in a similar manner as mammals, pain management in avians is none the less just as important. As with mammals, avians possess the necessary physiology to respond to the commonly available analgesics viz. opioids, sedatives, dissociative agents, local anaesthetics and the NSAIDs. From veterinary literature numerous NSAIDs that extend from aspirin to diclofenac have been recommended for the management of pain in various bird species[26,85]. Of these meloxicam, ketoprofen and carprofen have been considered the safest[25].

As seen with diclofenac, safety is a relative factor as certain species are more susceptible to toxicity than others. For example, extensive testing of flunixin in chickens, ducks, turkeys,

pigeons, and ostriches failed to reveal any serious side effects, while renal failure and finaly death were reported in three species of cranes[11,12,31]. Likewise the chicken appears to be susceptible to the effects of the NSAIDs, idomethacin and phenylbutazone[14,99].

2.5 Diclofenac

2.5.1 *Properties*

Diclofenac (2-[2-(2,6-dicholorophenyl amino)phenyl]acetic acid)(Figure 2-10) is a phenylacetic acid derivative that falls under the group of NSAIDs (MW 296.1g/Mol, CAS Registry: 15307-86-5)[87]. At present diclofenac features widely in human medicine due to its ability to manage osteoarthritis, inflammation and even gout (uricosuric)[29,112]. From a veterinary perspective diclofenac is used as an anti-inflammatory and analgesic drug in cattle.

At present there is no agreement on the sensitivity and specificity of diclofenac in inhibiting the COX enzyme in people. Goodman and Gilman suggest that it is a more COX II selective inhibitor but that prolonged use will result in the occurrence of gastric ulcers and thromboembolism[112]. As with the other NSAIDs, diclofenac is known to cause a number of adverse effects. It is known to be very ulcerogenic in both people and monogastric animals. Additionally diclofenac is also known to induce specific idiosyncratic cellular toxicity at the level of the liver and kidney in a small group of people.

Figure 2-10: Molecular structure of diclofenac

2.5.2 *Possible mechanisms of toxicity in vultures*

Although the vulture population decline has been conclusively linked to the veterinary use of diclofenac, the pharmacodynamic mechanism of toxicity resulting in the species crash has not yet been explained. With diffuse visceral gout being the only consistent finding during necropsies, it has been postulated that the kidney or its supportive vascular system are the site of toxicity[100,134]. Three hypotheses have been put forward as the possible pathophysiological mechanism of toxicity.

2.5.2.1 Ischaemic nephropathy with secondary visceral gout

Meteyer *et. al.* (2005) proposed that the nephrotoxicity results from the inhibition of renal prostaglandins and subsequent ischaemia[90]. Meteyer *et al.*. (2005) formulated this theory after observing atypical histopathological lesions, in vultures that had died from diclofenac toxicity. In these birds, the proximal convoluted tubules showed signs of necrosis in the absence of any urate deposits. This was contrary to the belief that toxicity was initiated by dehydration, subsequent build-up of uric acid and finally cellular damage. In their opinion Meteyer believed that the early changes in the mammalian nephron of the vulture kidney looked more like ischaemic damage as opposed to true necrosis. In their theory they suggested that an abnormally closed renal portal valve, induced by non-specific COX inhibition, alters oxygenation to such an extent that the resultant ischaemia promotes cellular damage and decreases uric acid clearance.

The renal portal valve, to which Meteyer refers, is a unique structure present in birds. In addition to the avian kidney, being composed of reptilian (Loopless Nephron) and mammalian nephrons (Looped Nephron), based on the absence or presence of the loop of Henle respectively, it is also unique because of its blood supply (Figure 2-11)[21]. While the mammalian kidney derives its entire blood supply from the efferent arterioles that arise from the renal artery, the looped nephron of the avian kidney receives a secondary blood supply from the hindquarters via the external iliac vein (Figure 2-12)[24,41,50,73,133].

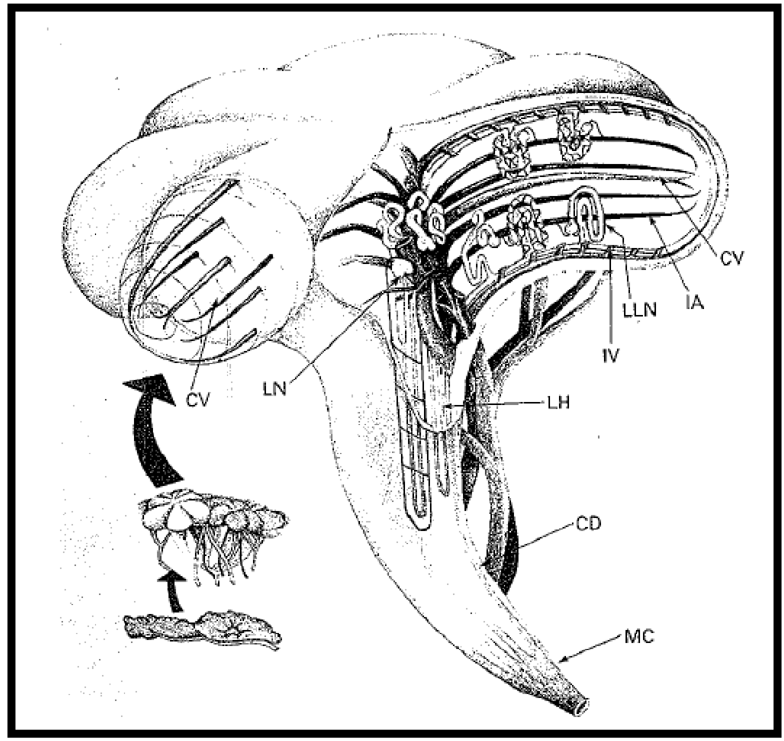


Figure 2-11: Illustration of the two different types of nephrons present in the avian kidney. Central vein (CV); Loopless nephron (LLN); looped nephrons (LN); loops of Henle (LH); collecting ducts (CD); medullary cones (MC); intralobular arteries (IA), interlobular/afferent veins (IV) [21]

It is within this portal circulation, known as the renal-portal circulation, that the renal portal valve (a physical valve) may be found. The valve, which anatomically may look like a real valve with cordae tendinae or a simple conical piece of muscle (Figure 2-13), may be found at various positions in the veins. In the chicken it is known to occur in at least five different sites within the portal blood vessels[24,73].

In birds the renal portal valve is believed to play an important role in regulating the blood supply to the kidneys under conditions characterised by stress. With the valve being innervated by muscarinic and beta receptors, conditions of stress characterised by the release of adrenaline leads to valve dilation, while acetylcholine promotes valve closure during normal physiological functioning[24,73,133]. When open, the valve allows blood from the external iliac vein to bypass the kidney and enter directly into the caudal vena cava. When closed, blood from the external iliac is no longer shunted and is free to enter into the cranial lobe of the kidney and support normal nephron functioning.

Meteyer linked the renal toxicity of diclofenac in people to that seen in the vulture. In mammals, the renal blood supply is controlled by the vascular tone of the afferent and efferent blood vessels. Under conditions characterised by stress or dehydration, the body maximises venous return to the heart by inducing vasoconstriction of peripheral vessels such as the rich renal vascular network. The endogenous ligands involved with this action include adrenaline, vasopressin and angiotensin II[8,40,96].

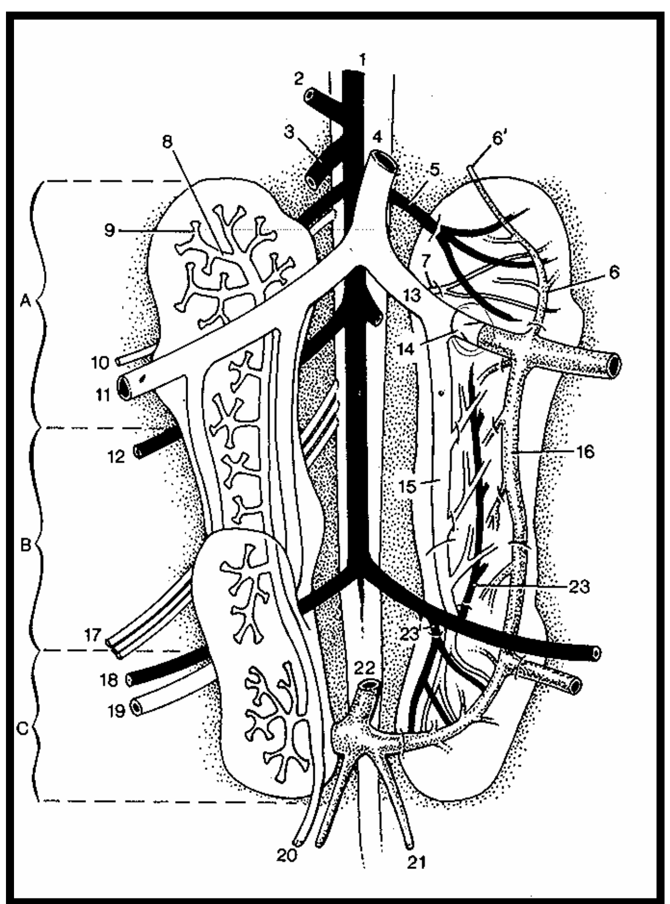


Figure 2-12: Illustration of the dual blood supply to the avian kidney

The right kidney shows the branches of the ureter; the left, the renal vessels. A, B, C, cranial, middle, and caudal divisions of kidney. 1, Aorta, 2, celiac a., 3, cranial mesenteric a.; 4, caudal vena cava; 5, cranial renal a.; 6, cranial renal portal v.; 6', anastomosis with vertebral venous sinus; 7, cranial renal V.; 8, primary branch of ureter; 9, secondary branch of ureter; 10, femoral n.; II, external iliac v.; 12, external iliac a.; 13, common iliac v.; 14, portal valve; 15, caudal renal v.; 16, caudal renal portal v.; 17, sciatic n.; 18, ischial a.; 19, ischial v.; 20, ureter; 21, internal iliac v.; 22, caudal mesenteric v.; 23, 23', middle and caudal renal aa.[41]

Since the induction of renal ischaemia is undesirable, complete vasoconstriction is prevented by the concurrent production of prostaglandin I_2 by the vascular smooth muscle, being stimulated by the relevant pressor[40]. This results in the coupled stimulation of the

prostaglandin receptors (Figure 2-14), thereby promoting vasodilation in the presence of the vasoconstrictor i.e. this modulates the degree of vasoconstriction. When diclofenac is present, the coupled pathway responsible for the formation of PGI_2 and PGE_2 gets inhibited, due to its COX inhibitory effect[22,65,82,96]. Without this modulatory pathway, the renal pressor induces complete vasoconstriction and renal ischaemic necrosis some time after initial exposure to the drug. Since uric acid can no longer be excreted it accumulates resulting in the clinical signs of hyperuricaemia and gout[50].

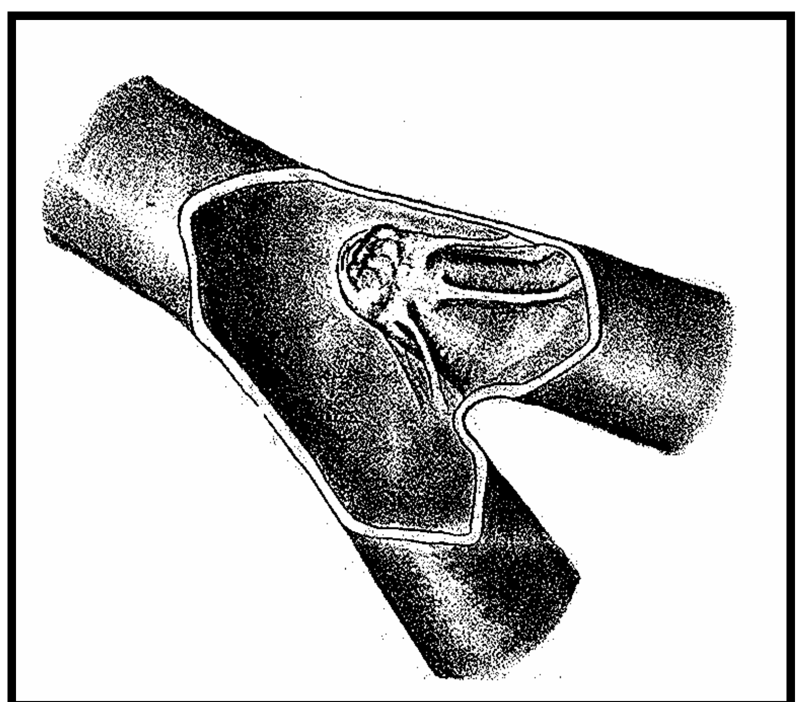
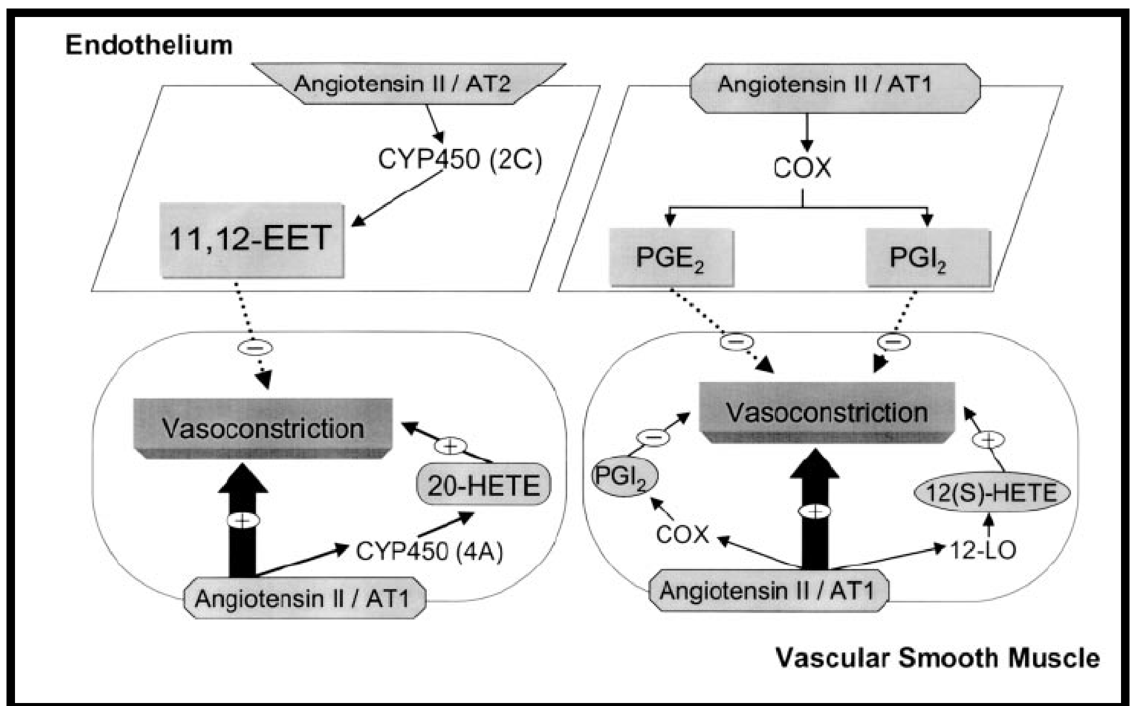


Figure 2-13: Illustration of the conical renal portal valve anchored to the mucosa by cordae tendinae[24]

Although Meteyer's theory is plausible it is not supported by certain physiological considerations. In studies undertaken in chickens, it has been shown that the valve is protective of kidney functioning, i.e. when the valve constricts blood enters into the cranial and caudal renal portal veins and supplies the kidney with portal blood. During conditions of stress, the valve opens and shunts blood from the external iliac directly into the caudal vena cava. Therefore, should diclofenac increase the contraction of the renal portal vein, through the inhibition of the PG synthesis, the valve should theoretically close and increase blood supply to the kidney.

Another problem associated with the theory is the assertion by Meteyer *et al.*., that the renal tubules receive their entire blood supply from the renal portal system. At present early studies on uric acid excretion indicate that the tubules have a dual blood supply from the renal artery and the portal vein[21]. It therefore is unlikely that closure of the renal portal valve could induce complete tubular ischaemia.



CYP450-Cytochrome P450, COX-Cyclooxygenase, PGE_2-Prostaglandin E_2, PGI_2-Prostaglandin I_2, EET- epoxyeicosatrienoic acid, HETE- hydroxyeicosatetraenoic acid, LO-Lipooxygenase; negative sign implies a modulatory effect, postive sign indicates smooth muscle contractions have been stimulated; AT_1 and AT_2 – are type 1 and type 2 angiotensin II receptors, respectively [65].

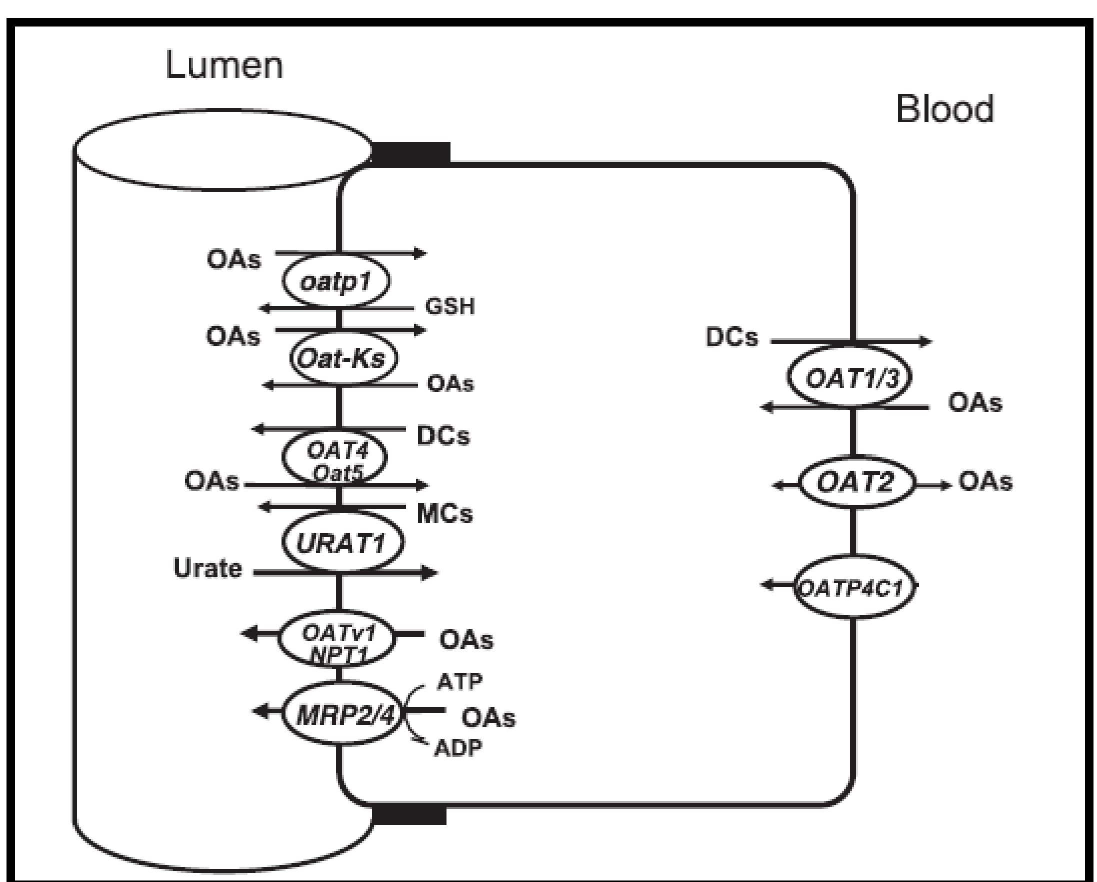
Figure 2-14: Illustration of the prostaglandin linked release following the stimulation of angiotensin receptors by AT2

2.5.2.2 Organic Anion Transporter antagonism

This is the first of two hypotheses proposed in this study. It has been hypothesized that gout is the result of the inhibition of the renal urate organic anion transporters (OAT) by diclofenac which subsequently leads to the clinical signs of hyperuracaemia and visceral gout in vultures.

From medical literature diclofenac is used to treat gout in people. Due to the importance of gout in people, a large amount of literature has been generated on the molecular mechanism of uric acid excretion[45,58,77,78,91,93,113,114,124,125,144]. This is best illustrated in the

Figure 2-15. The basolateral OATs (OAT 1,2 & 3), in contact with the blood vessel, are responsible for the active transport of uric acid from the efferent blood vessels into the intracellular environment. From here uric acid is excreted into the renal tubule by apical Multiple Resistance Protein (MRP) (MRP2 and MRP4) channels. In mammals uric acid may also be excreted into the tubules via filtration through the glomerulus. Once within the tubule uric acid may be excreted in the urine or it may conserved to variable degrees through reabsorption by the Uric Acid Transporter 1 (URAT1) channel.



OAs- Organic anions, DCs-dicarboxylates, MCs-monocarboxylates, OAT-various organic anionic transporters, URAT1-Urate transporter 1, MRP-Multiple drug resistant protein[10]

Figure 2-15: An overview of the molecular channels involved in the tubular excretion and reabsorption of uric acid in the nephron of man

With diclofenac being an important uricosuric drug in people, its mechanism of action has also been fully characterised[10,44,70,98]. The first channels to be inhibited by diclofenac are the basolateral OAT3 and OAT1 channels, which subsequently promote the build-up of

uric acid in the blood. Mammalian physiology hereafter prevents hyperuricaemia by increasing the glomerular excretion of uric acid. Until this point the amount of uric acud excreted by the body has not actually changed as the decreased excretion following channel inhibition has been compensated for by the increased glomerular filtration i.e. the same amount of uric acid is being excreted as during normal channel functioning. The actual increase in uric acid excretion comes through the concurrent inhibition of the URAT1 pump which prevents the reabsorption of uric acid. Since the uric acid can no longer be preserved, diclofenac promotes an increase in the excretion of uric acid in the body. The drug also has the ability to inhibit the apical MRP channel which is involved in the excretion of uric acid from within the cell[108].

The mechanism of uric acid excretion in birds differs from that of mammals. Birds are uricotelic in that they don't conserve uric acid[36,39,81,132]. Since the avian embryo develops in an egg, a very enclosed environment, the production of urea by the foetus can be harmful due to its dehydrating effect. To prevent dehydration the avian system has decreased the importance of urea as a nitrogenous waste product in preference of less dehydrating uric acid. Although the exact pathways involved in excretion are unknown in the vulture, a large body of literature is available for the chicken. This was mainly generated in an attempt to validate the chicken as a model for the study of new uricosuric agents for use in man.

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Chickens and probably all birds are different from man by being net uric acid excretors i.e. they minimally actively reabsorb uric acid[36]. They are similar to mammals in that the glomerulus is effective in the filtration of uric acid. None-the-less, the tubules still account for up to 75% of the excretion of uric acid[39,84]. In comparison to the transporters described in man above, chickens also make use of OAT1 & 3 channel to actively transport uric acid from the blood into the cell[39]. From the intracellular environment, uric acid is excreted into the tubule by the MRP2 & 4 channels. Hereafter, there is a difference between humans and birds, in that birds do not conserve uric acid by reabsorption, i.e. they do not possess a URAT1 channel[39].

At present the molecular effects of diclofenac on uric acid excretion have not been established in the chicken or any other bird. However, if one was to assume that the drug is

channel specific, it becomes plausible that diclofenac also inhibits the OAT3 pump in the vulture renal tubular epithelial cells. As seen in man, inhibition of this pump would result in an increase in uric acid in the blood, which should eventually produce the gout seen on necropsy.

A problem with this hypothesis is that it fails to explain the cellular damage, described by Meteyer *et. al.* (2005), i.e. cellular damage that precedes the formation of urate tophii. The hypothesis can however, be further modified by proposing that diclofenac inhibits the MRP channel, instead of OAT channels, in birds. This would initially promote the accumulation of uric acid within the cell, acidification of the intra-cellular environment, and subsequent cell death. Hereafter the non-excreted uric acid will accumulate and result in hyperuracaemia.

2.5.2.3 Secondary renal toxicity with or without toxic activation

The second hypothesis advanced relates to direct cellular toxicity and is based on the selected toxicity of diclofenac in a small percentage of the human population. At present diclofenac is a described toxin in the liver and kidneys, due to either direct drug toxicity or toxic metabolism, respectively. This therefore makes it plausible that toxicity could result at either organ system in the vulture.

2.5.2.3.1 Liver toxicity

Diclofenac is a known hepatotoxin in a select number of people due to the toxic activation of the molecule by the specific cytochrome, CYP2C9[19,52,102]. During phase I metabolism diclofenac gets converted by the enzyme into 5-OH-diclofenac and the minor metabolite, *N*,5$(OH)_2$-diclofenac. Hereafter the body enters into futile cycle in which the (*N*,5$(OH)_2$-diclofenac) gets continuously converted into 5-OH-diclofenac and vice versa resulting in the oxidation of NADPH by O_2. The reactive oxygen species (ROS) that forms subsequently decreases the selective permeability of the outer mitochondrial membrane [mitochondria membrane permeability (MMP)], most likely due to the oxidation of mitochondrial membrane proteins (**Figure 2-16**) [46,129]. Once exposed to the 5-OH metabolite calcium efflux also results from within the mitochondria which causes a further increase in ROS formation from an unknown mechanism[80].

Once the mitochondrial membrane is damaged, proteins (procaspases, caspase activators, and caspase-independent factors) efflux from within the mitochondria into the cytoplasm and promote the activation of Caspase 2, 8 and 9 and subsequently the cellular apoptosis cycle (Figure 2-17)[52,53]. The net effect is cellular death and a severe hepatitis in the susceptible person.

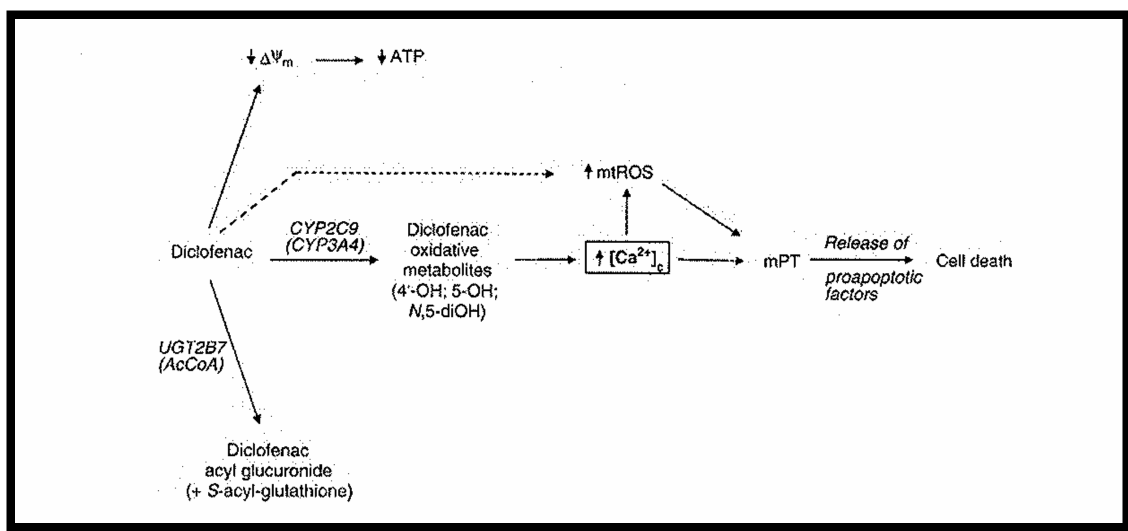


Figure 2-16: Illustration on how the metabolic activation of diclofenac leads to mitochondrial damage and apoptosis (Modified[80])

If toxicity is as a result of metabolic activation, it is possible that the vulture bio-activates diclofenac. This does, however, presume that toxicity starts within the liver or kidney and that renal effects are purely secondary, perhaps as a result from the accumulation of the active 5-OH-metabolite within the renal tubular epithelial (RTE) cells. The delay seen in the occurrence of toxic signs may therefore result from the slow metabolism of the drug.

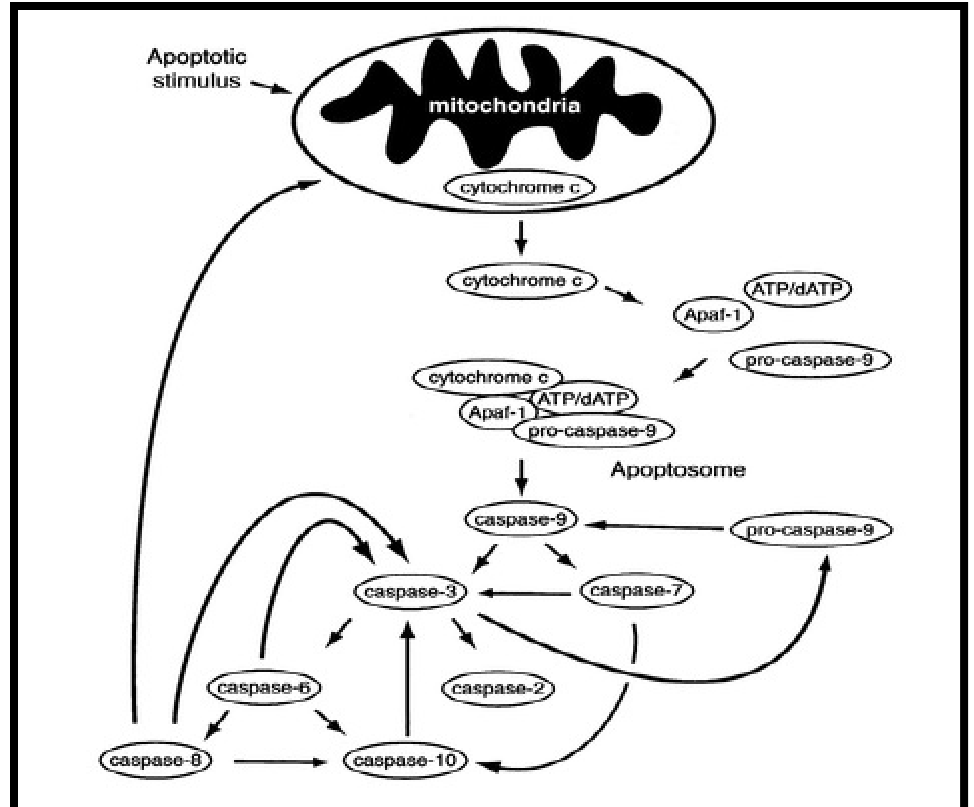
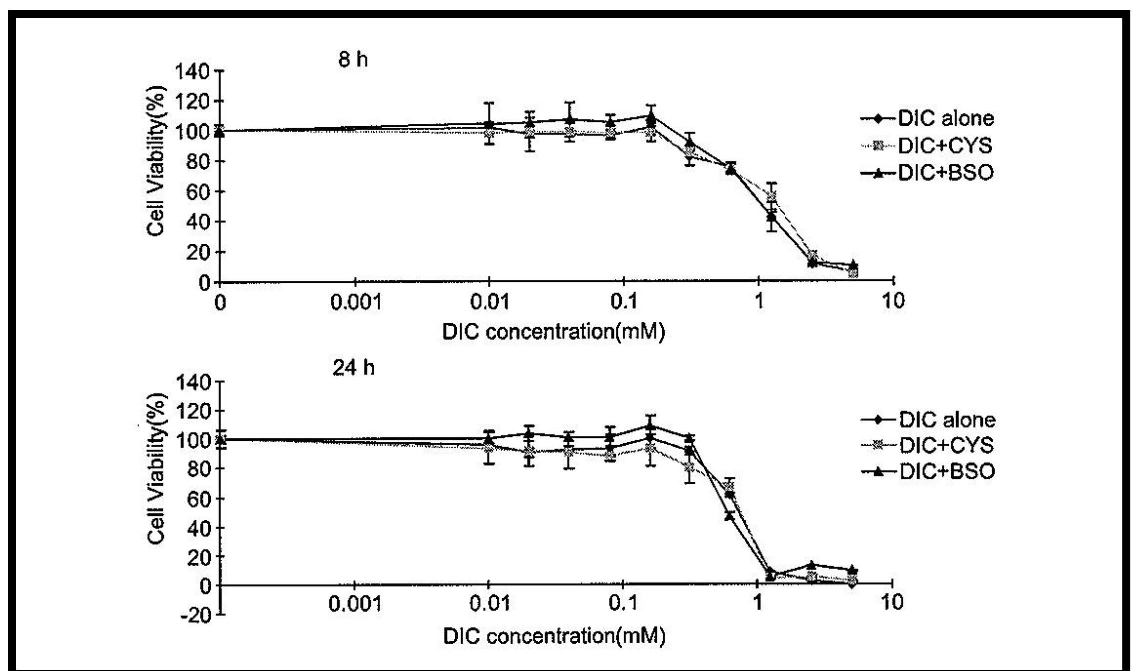


Figure 2-17: The pathways and enzymes involved in the activation of the caspase pathway and cellular apoptosis. Toxicity starts with the release of cytochrome C by the mitochondria with subsequent activation of the caspase pro-enzymes present in the cytoplasm[16]

2.5.2.3.2 Nephrotoxicity

Diclofenac is also known to be directly toxic to the RTE. In one assay cellular death was evident in rodent RTE cells at doses from 0.1 mM (Figure 2-18)[83]. In this specific assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was used to determine cellular viability at the level of the mitochondria. With the mitochondria being the only organelle in the mammalian cell with the ability to convert MTT to blue formazin, the failure in conversion as reported in the study was therefore indicative of mitochondrial toxicity.



The CYS and BSO curves were added studies under to demonstrate that these substances did not play a role in the toxicity of diclofenac

Figure 2-18: Cell viability dose response curve of renal tubular epithelial cells incubated with diclofenac for 8 and 24 hours. Also included are the curves illustrating the effect of co-incubation with cysteine (CYS) and DL-buthionine-(S,R)-sulfoximine (BSO)[83]

As in the liver, mitochondrial oxidative damage and NADPH depletion is believed to be the cause of cellular death[60,97]. However the description of the pathophysiology in the kidney is incomplete since the mechanism of the oxidative injury within the RTE cell is unknown. It has, however, been demonstrated that diclofenac induces the efflux of calcium from the RTE mitochondria.[103,104] Once the mitochondrion is damaged, following exposure to diclofenac, the RTE cell is unable to move malate and glutamate into the mitochondria[97]. In the absence of these two products, the mitochondrial electron transport chain fails with a resultant decrease in the formation NADPH. With cellular respiration shutting down, ATP generation no longer occurs, with the end result being the mitochondrial activation of the caspase pathways as described above.

From the direct RTE toxic effect of the drug, it is possible that diclofenac toxicity in the vulture is due to direct cellular toxicity in the kidney. It has also been suggested that a

suppression of ATP generation results in an inhibition of the MRP pump with a subsequent build-up of uric acid within the cell[97]. Although this theory will best explain the renal toxicity being seen, it fails to explain why toxicity is delayed for between 24-36 hours from initial exposure. From the cell cultures using the rodent cells, toxicity was evident as early as 8 hours post cellular exposure at high concentrations[83].

2.6 Towards the protection of a disappearing species

2.6.1 *Steps necessary to protect the species*

From the problems associated with the crash in the vulture population, both national and international organisations met in an attempt to prevent a species' extinction. At these international meetings on the Asian vulture crisis (the Kathmandu Summit Meeting on the veterinary use of the drug diclofenac, Kathmandu, 5-6 February 2004 and the South Asian Vulture Recovery Plan Workshop, India, 12-14 February 2004) certain conservation action plans for the Asian Gyps species were put forward to promote the future survival of the species. They are summarised as follows:

- It is essential to establish viable captive populations of all three threatened Asian vulture species.

- It is necessary to control the veterinary use of diclofenac so as to remove it as a contaminant of the food of wild vultures.

- In addition potential alternatives to diclofenac in these Asian countries will need to be identified.

2.6.2 *Conservation Efforts: Establishment of a captive population*

At present the establishment of breeding centres fall under the auspices of the Royal Society for the Protection of Birds (RSPB). In their efforts, numerous breeding centres were established in various parts of India, and populated with captive breeding pairs. One

of the problems of this system, and thus repopulating the species, is the slow breeding habit of the vulture. Being such long lived birds, vultures only reach breeding maturity at approximately seven years of age[95]. Added to this, vulture pairs only produce one egg a year, of which survival is not always guaranteed. Thus even if the breeding centres are successful, it will never be able to return the population to its previous abundance. All they may do will be to prevent the species extinction. It therefore becomes imperative that the current Asian vulture population is prevented from further declines. Although the latter would be best implemented by banning the manufacture and sale of diclofenac, the Indian government was reluctant to issue this mandate due to the possible suffering that could result in cattle from the absence of an effective analgesic agent. They did, however, issue a compromise in that they promised to ban diclofenac if a suitable alternate NSAID, both vulture safe while still being effective in cattle, could be identified[135]. Therefore for diclofenac to be removed from the veterinary market and therefore the vulture food chain, a suitable replacement needed to be identified.

2.6.3 *Removal of diclofenac from the food chain*

To identify this safe drug, survey forms were circulated by the RSPB to all wildlife veterinarians, zoos, and rehabilitation centres that had any experience in managing pain in vulture using the NSAIDs[35]. From this survey, reports on the treatment of over 870 vultures and scavenging birds of 79 species were collected. Diclofenac, carprofen, ibuprofen, phenylbutazone and flunixin were identified as potentially toxic (Table 2-1). In contrast meloxicam, a newer oxicam, appeared to be completely safe in over 700 raptors and scavenging birds representing 60 different species.

Although meloxicam appeared to be the ideal alternate from the survey its safety in vultures needed to be conclusively determined. An undertaking that is unfortunately, rather complex as statistically robust toxicity studies needed to be undertaken to prove their safety. With the three affected Asian species already being rare and critically endangered and few non-releasable birds being available for toxicity testing, an alternative model needed to be described for future testing. With the closest apparent relative, and therefore the most likely surrogate for the Asian white-backed vulture being the African White-back Vulture (AWBV), Swan and coworkers (2006) exposed two non-releasable

AWBV to diclofenac at a dose of 0.8 mg/kg[134]. In this study the authors were able to show that the AWBV was at least (or possibly more) susceptible to toxicity as their oriental cousins, and therefore more than adequate for future safety studies. The work undertaken by Swan and coworkers[134], was the first step in finding a suitable alternate drug and characterising the mechanism of toxicity of diclofenac in susceptible vultures.

Table 2-1: Survey results from the RSPB study indicating the number of animals and safety of NSAIDs in various vulture species

Drug	**Toxicity**	**n**	**Dose (mg/kg/bw)**	**Species treated**
Aspirin	No	3	5.4 to 6.4	*Aegypius monachus, Ciconia ciconia, Corvus corax*
Ketoprofen	No	20	1.0 to 7.7	*Gyps fulvu, Gyps rueppellii, Aegypius monachus, Necrosyrtes monachus, Buteo jamiacensis, Geranoaetus melanoleucus, Vultur gryphus, Leptoptilos crumeniferus, flammeus flammeus, Bubo virginianus, Otus asio*
Meloxicam	No	739	0.1 to 0.75	34 species in total were listed as being treated, of which four species were old world vultures (46) including Gyps species (n=39) and for new world vulture species (n = 21)
Ketoprofen & Meloxicam	No	1	Ket 1.0, Mel 0.2	*Gyps africanus*
Carprofen	Yes	5	1.0 to 5.0	*Gyps fulvus, Parabuteo unicinctu, Aegolius acadicus*
Carprofen	No	35	1.5 to 7.6	*Gyps africanus, Gyps bengalensis, Gyps fulvus, Gyps himalayensis,Gyps africanus, Aegypius monachus, Necrosyrtes monachus,Haliaeetus leucocephalus, Ciconia ciconia, Ephippiorhynchus senegalensis, Bugeranus carunculatus, Grus vipio, Ardeotis kori*
Diclofenac	Yes	28	0.1 to 2.5	*Gyp bengalensis, Gyps africanus, Gyps fulvus*
Diclofenac	No	8	0.25 to 0.6	*Gyp bengalensis*
Flunixin	Yes	7	1.0 to 4.5	*Gyps rueppellii, Cariana cristata, Leptoptilos crumeniferus, Platalea alba, Aegypius monachus*
Flunixin	No	16	0.5 to 12.0	*Gyps fulvus, Gyps rueppellii, Haliaeetus leucocephalus, Terathopius ecaudatus, Parabuteo unicinctus, Leptoptilos crumeniferus, Aegypius monachus, Vultur gryphus*
Ibuprofen	Yes	1	-	*Aegypius monachus*
Phenylbutazone	Yes	1	-	*Torgus tracheliotus*
Flunixin or Ketoprofen	Yes	1	-	*Gyps africanus*
Carprofen & Ketoprofen	Yes	1	Car 7.2, Ket 4.3	*Gyps africanus*

Modified from Cuthbert *et al.*, 2006

2.6.4 *The safety of other NSAIDs*

Unfortunately with diclofenac proving to be so toxic, questions on the safety of all other veterinary NSAIDs have already been raised[7]. With numerous different NSAIDs being available for veterinary use, in different parts of the world, it is possible that other vulture species and potentially other bird species could face similar population declines following their exposure to a NSAID. To satisfactory answer this question toxicity testing will have to be undertaken on all available NSAIDs. Once again an undertaking that is very complex, as it is impossible to test all these drugs in wild vultures, especially since the population of the AWBV has also been declining in numbers. As a result of the improbability of *in vivo* testing in the target species, from both an ethical and financial standpoint, it would be preferable for an *in vitro* model to be developed to establish the degree of toxicity. To develop such a model the mechanism of toxicity of diclofenac must first be established. Alternatively it may also be possible to find a more commonly available domestic bird species as a surrogate model.

2.7 Conclusion

It has been conclusively proven that diclofenac is toxic to Asian white-back vultures. Although, the environmental devastation may never be reversed, it is imperative that the extinction of the species is prevented. At present the breeding facilities set up in India are only one option of achieving this. A potentially better option would be to prevent further declines in the current population. Since this would entail a ban on the sale and use of diclofenac in domestic stock, a vulture-safe NSAID, for use in stock needs to be identified. With a model already being validated by Swan and coworkers., further toxicity testing of this alternate is now possible.

The eventual validation of a model for the further toxicity screening of other NSAIDs is needed. In addition the mechanism of diclofenac's toxicity needs to be established in order to establish a laboratory bench model.

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CHAPTER 3: Removing the Threat of Diclofenac to Critically Endangered Asian Vultures

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REMOVING THE THREAT OF DICLOFENAC TO CRITICALLY ENDANGERED ASIAN VULTURES

Gerry Swan[1], Vinasan Naidoo[1], Richard Cuthbert[2], Rhys E. Green[2,3], Deborah J. Pain[2], Devendra Swarup[4], Vibhu Prakash[5], Mark Taggart[6], Lizette Bekker[1], Devojit Das[5], Jörg Diekmann[7], Maria Diekmann[7], Elmarié Killian[1], Andy Meharg[6], Ramesh Chandra Patra[4], Mohini Saini[4], Kerri Wolter[8]

1 Department of Paraclinical Sciences and Biomedical Research Centre, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa, **2** Royal Society for the Protection of Birds, The Lodge, Sandy, Bedfordshire, United Kingdom, **3** Conservation Biology Group, Department of Zoology, University of Cambridge, Downing Street, Cambridge, United Kingdom, **4** Indian Veterinary Research Institute, Izatnagar 243122, Uttar Pradesh, India, **5** Bombay Natural History Society, Hornbill House, S.B. Singh Road, Mumbai, India, **6** School of Biological Sciences, Department of Plant and Soil Science, University of Aberdeen, St Machar Drive, Aberdeen, United Kingdom, **7** Rare and Endangered Species Trust, PO Box 178, Otjiwarongo, Namibia, **8** Vulture Unit, De Wildt Cheetah and Wildlife Trust, PO Box 1756, Hartbeespoort, South Africa

3.1 Abstract

Veterinary use of the non-steroidal anti-inflammatory (NSAID) drug diclofenac in South Asia has resulted in the collapse of populations of three vulture species of the genus *Gyps* to the most severe category of risk of global extinction. Vultures are exposed to diclofenac when scavenging on livestock treated with the drug shortly before death. Diclofenac causes kidney damage, increased serum uric acid concentrations, visceral gout and death. Concern about this issue led the Indian Government to announce its intention to ban the veterinary use of diclofenac by September 2005. Implementation of a ban is still in progress late in 2005, and to facilitate this we sought potential alternative NSAIDs by obtaining information from captive bird collections worldwide. We found that the NSAID meloxicam had been administered to 35 captive *Gyps* vultures with no apparent ill effects. We then undertook a phased programme of safety testing of meloxicam on the African white-backed vulture *Gyps africanus*, which we had previously established to be as susceptible to diclofenac poisoning as the endangered Asian *Gyps* vultures. We estimated the likely maximum level of exposure (MLE) of wild vultures and dosed birds by gavage (oral administration) with increasing quantities of the drug until the likely MLE was exceeded in a sample of 40 *G. africanus*. Subsequently, six *G. africanus* were fed tissues from cattle which had been treated with a higher than standard veterinary course of

meloxicam prior to death. In the final phase, ten Asian vultures of two of the endangered species (*G. bengalensis*, *G. indicus*) were dosed with meloxicam by gavage; five of them at more than the likely MLE dosage. All meloxicam-treated birds survived all treatments, and none suffered any obvious clinical effects. Serum uric acid concentrations remained within the normal limits throughout, and were significantly lower than those from birds treated with diclofenac in other studies. We conclude that meloxicam is of low toxicity to *Gyps* vultures and its use in place of diclofenac would reduce vulture mortality substantially in the Indian subcontinent. Meloxicam is already available for veterinary use in India.

3.2 Introduction

Veterinary use of the non-steroidal anti-inflammatory drug diclofenac is a major cause of the catastrophic collapse of *Gyps* vulture populations in the Indian sub-continent [1-3]. Three species of vultures endemic to South Asia, which together used to number tens of millions, are now at high risk of global extinction and are listed as Critically Endangered [4]. Populations of Oriental white-backed (*Gyps bengalensis*), long-billed (*G. indicus*) and slender-billed vultures (*G. tenuirostris*) have declined by more than 95% since the early 1990s [5,6], and continue to decline at an annual rate of 22% to 48% [3].

Diclofenac is a widely available veterinary drug in the Indian sub-continent, where it is used for the symptomatic treatment and management of inflammation, fever and/or pain associated with disease or injury in domestic livestock. Vultures are exposed to the drug when they consume carcasses of cattle that were treated with diclofenac shortly before death. Following experimental exposure to diclofenac or diclofenac-contaminated tissues, *Gyps* vultures die within days from kidney failure with clinical signs of extensive visceral gout (formation of uric acid crystals within tissue) [1,7]. These clinical signs and diclofenac residues in vulture tissues have been found in carcasses of wild *Gyps* vultures from across India, Pakistan and Nepal [1,2], and the proportion of vulture carcasses with signs of diclofenac poisoning is consistent with this being the main, and possibly the only, cause of the vulture decline [3].

The loss of tens of millions of vultures over the last decade has had major ecological consequences across the Indian subcontinent that pose a potential threat to human health.

In many places, populations of feral dogs (*Canis familiaris*) have benefited from the disappearance of *Gyps* vultures as the main scavenger of wild and domestic ungulate carcasses [8]. Associated with the rise in dog numbers [9] is an increased risk of human cases of rabies. If rat (*Rattus* spp.) populations also increase at carcass dumps in and near settlements, the risk of transmission of diseases including bubonic plague to humans may also increase. Vultures probably also helped to control livestock diseases, such as brucellosis, tuberculosis and anthrax by disposing of infected carcasses [10, 11]. The loss of vultures has had a social impact on the Indian Zoroastrian Parsi community, who have traditionally utilized vultures to dispose of human corpses in "sky burials" [12] and are now having to seek alternative disposal methods [13]. As a consequence of the collapse of vulture populations, national and international conservation organisations have concluded that it is essential to ban the use of diclofenac in livestock so as to remove it as a contaminant of the food of wild vultures [14]. At a Meeting of the National Wildlife Board in March 2005, the Government of India announced that they intended phasing out the veterinary use of diclofenac [15].

The identification of alternative non-steroidal anti-inflammatory drugs (NSAIDs) that are effective for the treatment of livestock, but also relatively non-toxic to vultures, would facilitate the removal of diclofenac from the food of vultures. NSAIDs are characterised by their ability to inhibit cyclo-oxygenase enzymes, which are involved in the formation of prostaglandins. However, there are marked differences between drugs in their selective inhibition of the two sub-types of cyclo-oxygenase COX-1 and COX-2, with the latter being involved with the modulation of inflammatory responses and pain, while the former modulates blood flow to the kidneys. The ability of NSAIDs to inhibit both these subtypes has been implicated as a cause of the severe side effects occasionally associated with the use of some NSAIDs [16]. Toxic effects on the kidneys of birds have been observed following treatment with a number of NSAIDs [1,17]. However, there are marked inter-specific differences in toxicity [18-20] and it is necessary to establish the safety of individual NSAIDs to *Gyps* vultures. To identify candidate alternative drugs, we contacted veterinarians at zoos and wildlife rehabilitation centres worldwide, and requested information on the clinical use of NSAIDs on captive *Gyps* vultures, including the outcome of such treatment. Preliminary results suggested that the NSAID meloxicam is a potential alternative for diclofenac, because 35 individuals from six *Gyps* species (including five

Oriental white-backed vultures) treated with meloxicam, typically at doses of 0.2-0.5 mg kg^{-1}, showed no ill effects; whilst the use of several other NSAIDs was associated with renal failure (RSPB and NBPT unpublished data).

As all three of the resident Asian *Gyps* vultures are Critically Endangered, we considered it unacceptable to use these species for safety testing without first evaluating the safety of meloxicam on a suitable surrogate. The African white-backed vulture (*G. africanus*) was chosen as a surrogate because it has a favourable global conservation status (category Least Concern) [4] and diclofenac has been shown experimentally to be as toxic to it as it is to the endangered *G. bengalensis* [7]. Clinical signs at post-mortem examination of experimentally dosed birds indicate a similar mechanism of toxicity in both species. Diclofenac-dosed *G. africanus* showed significant increases in serum uric acid concentrations 12-24 hours after dosing and exhibited lethargy and neck-drooping behaviour before death [7].

In this paper, we report tests on the safety of meloxicam to *Gyps* vultures, which we dosed with meloxicam by gavage (oral administration) and by feeding them with tissue from meloxicam-dosed cattle. With both routes of drug administration, the range of dose levels we used exceeded our estimated likely maximum level of exposure (MLE) of meloxicam to wild vultures. To minimise the risk of suffering and death of experimental animals, safety testing was undertaken in six phases (summarised in Table 1). During the first three phases, the dose rate of meloxicam administered by gavage to *G. africanus* was progressively increased from 0.5 mg kg^{-1} vulture body weight to 1 mg kg^{-1} and then to the highest dose of 2 mg kg^{-1}, which exceeds our estimate of the MLE (Protocol S1). At the conclusion of each phase the results were evaluated and the study only proceeded to the next phase if all of the dosed birds were healthy and had clinically normal serum concentrations of uric acid and alanine transferase (ALT), both of which are known to be elevated beyond the normal range in *G. africanus* after treatment with diclofenac [7]. In the fourth phase, meloxicam was administered at 2 mg kg^{-1} to captive *G. africanus* in South Africa and wild vultures in Namibia, thereby exposing a larger number of vultures from two distinct populations to the estimated MLE of meloxicam in the wild. The fifth phase of the study simulated the natural route of NSAID exposure, by feeding vultures with liver and muscle tissue from cattle that had received a higher than standard veterinary course of

meloxicam treatment, with daily injections over five days. The final phase of testing was to assess the safety of meloxicam to two of the three critically endangered Asian vultures, by administering meloxicam by gavage to captive *G. bengalensis* and *G. indicus* in India.

3.3 Results and Discussion

3.3.1 *Phases I-III: Safety testing using captive G. africanus*

In each of the first three phases of our study, we administered a single dose of meloxicam to five vultures by gavage (oral administration into the crop via a five mm tube) and gave sterilised water to three control birds by the same method. The birds' apparent health and serum parameters were then assessed for seven days after treatment. Dose rates in Phases I to III were 0.5, 1 or 2 mg kg^{-1} respectively, and were set so that the highest dose just exceeded the likely MLE of wild vultures (estimated as 1.83 mg kg^{-1} vulture body weight; Protocol S1). No ill-health was observed in any of the 15 vultures treated with meloxicam at these three dose levels and all birds were alive and healthy at the end of the experimental period (Table 3-2). There was a significant loss of body mass during the experimental period in Phases I, II and III (matched pairs *t* test; Phase I $t_7 = 7.28$, $p < 0.001$; Phase II $t_7 = 2.97$, $p < 0.05$; Phase III $t_7 = 2.96$, $p < 0.05$). However, there was no significant difference between the meloxicam dosed and control birds in body mass change as a percentage of initial mass in any of the three Phases (2-sample *t* test; Phase I $t_6 = 0.13$, $p > 0.89$; Phase II $t_6 = 0.46$, $p > 0.66$; Phase III $t_6 = 0.61$, $p > 0.56$). Because of this, and because no significant loss of body mass was observed in later phases of the experiment, when birds were handled for sampling on fewer occasions and not moved from their normal holding aviaries (see below), we believe that the loss of body mass was most likely due to the stress caused by handling and sampling, rather than by meloxicam.

We compared the survival of vultures in these experiments with that of two *G. africanus* treated with comparable doses of diclofenac using the same methods [7]. In each phase, all five meloxicam-treated vultures survived the experimental period, whereas both diclofenac-treated birds died with extensive visceral gout. This represents a statistically significant difference in death rate between the two drugs (2-tailed Fisher exact test; 0/5 deaths versus 2/2 deaths, $p = 0.0476$ in each phase). However, because of the small sample sizes, these results do not exclude the possibility that, in a worst-case scenario, meloxicam

might have caused appreciable mortality if used on a larger sample. For example, with a total sample of 15 treated birds there still could statistically be a 5% chance of no birds dying, even if the true probability of death per trial was as high as 18% ($(1\text{-}0.18)^{15} = 0.05$). If only the five birds treated in Phase III with more than the MLE are considered, the failure to observe any deaths implies that there could be a 5% probability that the true risk of death per trial might be as high as 45% ($(1\text{-}0.45)^{5} = 0.05$), which led us to test a larger sample of birds in Phases IV and V (see below).

Although the survival of all of the meloxicam-treated vultures in Phases I-III is not robust evidence of safety on its own, it can be combined with information obtained by sampling the blood of experimental and control birds. There were no significant differences in serum concentrations of uric acid, ALT, albumin and creatinine kinase between treated and control groups in any of the three phases and for any of the sampling times after dosing (Table 3-2). Inspection of the magnitude of average differences in serum concentrations between treated and untreated birds showed no indication of a systematic trend for any of the serum constituents in relation to dose (Figure 3-1, Table 3-2). Since the serum concentration of uric acid has been shown to be elevated well beyond the normal range in *G. africanus*, *G.bengalensis* and *G. fulvus* treated with comparable, fatal doses of diclofenac [1,7], these observations provide substantial further evidence of safety.

3.3.2 *Phase IV: Safety testing using larger numbers of captive and wild-caught G. africanus*

Our objective in the next phase of the study was to narrow the range of possible values of the true rate of meloxicam-induced mortality that would be consistent with our data by testing larger numbers of vultures with more than the likely MLE. In this phase, we treated two groups of *G. africanus*. In Phase IV.1 we used 14 long-term captive birds that had been used more than six weeks previously in Phases I to III (11 as experimental birds and three as controls). We treated all 14 birds with meloxicam. In Phase IV.2 we captured 25 wild *G. africanus* in Namibia and held them temporarily. Of these birds, 21 were treated with meloxicam and four received sterilised water and acted as controls. All treated birds in Phase IV were given 2 mg kg^{-1} of meloxicam by gavage (Table 3-1).

All 35 meloxicam-treated birds survived the seven-day experimental period and the wild-caught vultures used in Phase IV.2 were all successfully released after the experiment. There was no significant change in the body mass of meloxicam-treated birds between the beginning and end of the seven-day period for either captive (matched pairs t test; $t_{13} = 0.29$, $p > 0.77$) or wild caught birds (matched pairs t test; $t_{24} = 1.68$, $p > 0.10$). For the wild-caught birds there was also no significant difference in the percentage mass change of meloxicam dosed and control birds (2-sample t test; Phase I $t_{23} = 0.30$, $p > 0.77$). Serum uric acid concentrations did not differ significantly between experimental and control groups and showed no trend during the experimental period (Table 3-2, Figure 3-1). Neck-drooping behaviour, similar to that seen in diclofenac dosed birds [7], was observed in the Phase IV.2 birds soon after the collection of the second blood sample at 48 hours following treatment, and two birds lay on the ground. However, neck-drooping was observed in both meloxicam-dosed and control birds, and occurred during the heat of the day. Hence, we consider that the neck-drooping we observed was most likely to be a thermoregulatory activity [21] in response to high ambient temperature and an elevation of body temperature caused by the stress of handling and sampling, rather than a response to meloxicam treatment. By the end of the day, all birds (including the two recumbent birds) had resumed a normal body posture. Neck-drooping was not observed over the remaining five days of the trial. Hence, we consider it to be a non-specific response to stress caused by heat or handling and not a specific response to NSAID poisoning.

When the results from Phases III and IV of the study are combined, 40 *G. africanus* were treated by gavage with more than the likely MLE of meloxicam and all survived with no ill-effects observed that were attributable to the drug. These data indicate a 95% probability that the true probability of death per trial consistent with these data was no higher than 7% ($(1-0.07)^{40} = 0.05$). Taken together with the evidence of lack of an effect of meloxicam on serum uric acid concentrations, these results indicate that meloxicam administered by gavage does not cause appreciable mortality in *G. africanus*.

3.3.3 *Phase V: Safety testing by feeding G. africanus on tissues of meloxicam-treated cattle*

We wished to assess the possibility that, although meloxicam itself appears safe when administered to vultures at the MLE, metabolites produced by treated cattle might be toxic. To test this, we gave daily injections of 1.0 mg kg^{-1} of meloxicam to three cattle (*Bos taurus*) for five days. This is a higher dose level than the two standard veterinary doses recommended in India (0.5 to 0.7 mg kg^{-1} daily for five consecutive days). We slaughtered the three cattle eight hours after the last injection and fed liver or muscle to six captive *G. africanus*. An experiment by EMEA on *Bos taurus* found that tissue meloxicam concentrations in treated animals were higher in liver than other tissues tested, and peaked at the 8 hour sampling period (Protocol S1) [20]. In our experiment, concentrations of parent meloxicam in cattle tissues at slaughter averaged 0.50 ± 0.13 (± 1 standard deviation) mg kg^{-1} for muscle and 8.12 ± 1.10 mg kg^{-1} for liver. Vultures consumed an average of 0.59 ± 0.21 (± 1 standard deviation) kg of liver and 0.67 ± 0.32 kg of muscle tissue, of the 1 kg with which they were each provided, within the 48 hour feeding period. On one occasion, a bird ate all of the liver provided and on two occasions, birds ate the entire portion of muscle. The dose of parent meloxicam ingested ranged from 0.03-0.15 mg kg^{-1} vulture body weight for muscle, and from 0.57-1.98 mg kg^{-1} body weight for birds feeding on liver. Because we administered meloxicam for five days at a higher dose (1.0 mg kg^{-1}) than in the EMEA study (0.7 mg kg^{-1}) [22], the maximum dose ingested by a vulture (1.98 mg kg^{-1} bw) and the maximum cattle liver tissue concentrations (8.91 mg kg^{-1}) are somewhat higher than those predicted from the EMEA work (Protocol S1). For comparison, we also administered meloxicam by gavage at doses (1.18 to 2.45 mg kg^{-1} vulture body weight) intended to be similar to those ingested by birds feeding on liver. All six birds survived the treatments and no ill-effects or altered feeding behaviour was observed. There was no significant change in body mass between the start and end of the five-day experimental period for any of the three treatment types (matched pairs *t* test; muscle $t_5 = 1.00$, $p > 0.36$; liver $t_5 = 2.44$, $p > 0.05$; gavage $t_5 = 1.46$, $p > 0.20$). Serum uric acid concentrations remained within the 95% range observed in these individuals before treatment at both sampling times and also within the similar 95% ranges for uric acid for

wild *G. africanus* captured in Namibia and reported for *G. africanus* captured in Kenya [23] (Figure 3-2). There was no significant relationship between uric acid concentration and meloxicam dose at 48 h or 96 h (OLS regressions of log uric acid concentration on log meloxicam dose for each of the three administration routes; $p > 0.05$ in all cases). This was also the case when the log of the ratio of the uric acid concentration after treatment to that before treatment was used as the dependent variable. A more elaborate analysis of variance in which log uric acid concentration was modelled as a function of treatment method, time period and log meloxicam dose, with pre-treatment log uric acid concentration as a covariate, also gave no indication of any significant effect on serum uric concentration of treatment with meloxicam by any of the three routes (Protocol S2). The absence of mortality or elevation of serum uric acid levels indicates that tissues of cattle treated with meloxicam shortly before death are unlikely to be toxic to *G. africanus*. The experiments using liver tissue are particularly informative, because the quantity of liver eaten by one bird approached the maximum meal size likely to be consumed by a wild vulture and this bird received a dose of parent meloxicam in excess of the likely MLE.

3.3.4 *Phase VI: Safety testing of meloxicam on Endangered Asian Gyps*

Although the experiments we have reported so far indicate that meloxicam appears safe for *G. africanus*, this does not exclude the possibility that it might be toxic to Asian *Gyps* species, though this seems unlikely in view of the close phylogenetic relationships within the genus [24] and the similarity of the response to diclofenac of *G. africanus* and *G. bengalensis*. We therefore administered meloxicam doses of 0.5 mg kg^{-1} by gavage to three captive *G. bengalensis* and two *G. indicus* and the MLE of 2.0 mg kg^{-1} to three *G. bengalensis* and two *G. indicus*. All 10 meloxicam-treated birds survived the seven-day experimental period and they remain alive and healthy four months afterwards. None showed signs of ill-health or abnormal behaviour. There was no significant change in body mass during the experimental period (paired t-test; $t_5 = 2.07$, $p > 0.09$).

Hence, although the number of birds tested was small, there is no indication of adverse effects of meloxicam on these two species of Asian *Gyps* vultures.

3.4 Conclusions

The results of this study demonstrate that meloxicam is much less toxic than diclofenac in at least three *Gyps* species, including two of the Critically Endangered Asian species. Indeed, we found no evidence that meloxicam administered at doses exceeding our estimated likely maximum level of exposure caused any deaths or even elevation of serum uric acid concentrations. Combining the results of this study with those from the questionnaire to zoo veterinarians, a total of at least 88 individual birds from seven *Gyps* species are known to have received meloxicam at various doses with no recognized adverse effects. Hence, with this total of treated birds there is a 95% chance that the per trial probability of mortality caused by meloxicam is no higher than 3.5%. The observation that serum concentrations of uric acid remain within the normal range for all meloxicam dose rates adds substantially to the evidence that meloxicam has low toxicity to *G. africanus*, given that uric acid concentrations in this and two other *Gyps* species were markedly elevated by lethal treatment with diclofenac [1,7]. Preliminary results from the NSAID questionnaires indicate the safety of meloxicam to a wide range of other vultures, raptors and scavenging bird species, and to date we know of over 700 individuals from more than 30 species that have been treated with no apparent adverse affects (RSPB and NBPC unpublished data). This demonstrates that, at recommended clinical dose levels, meloxicam is not toxic to a wide range of avian species.

Any replacement for diclofenac must be effective for the treatment of livestock as well as safe for vultures. Meloxicam is one of the newer NSAIDs with preferential COX-2 inhibition, having analgesic, antipyretic and anti-inflammatory properties and a reduced risk of adverse effect on renal function [16,25]. It is used to treat a variety of veterinary ailments [26-30], and it is rated as a highly effective NSAID [30-32]. Meloxicam is approved for human use in more than 80 countries including India [33, 34]. It is used and licensed as a veterinary drug in India, Europe and North America [35,36] and is already manufactured in India, where, like diclofenac, it is available as both an injectable solution and oral bolus. We hope that efforts to prevent diclofenac being used to treat domestic livestock in the Indian subcontinent and in other *Gyps* vulture range states will continue as a matter of urgency. Where the availability of alternative drugs is seen as a barrier to achieving this objective, we recommend that governments consider advocating the use of

meloxicam as an alternative to diclofenac. Because vulture populations are now very low and contamination of even a small proportion of livestock carcasses is sufficient to cause adverse impacts on vulture populations [3] we also advocate immediate intensification of efforts to establish viable captive populations of all three Critically Endangered species.

3.5 Materials and Methods

Trial Animals: Non-releasable captive vultures held at the De Wildt Cheetah and Wildlife Trust (South Africa) were used for Phases I-III, Phase IV.1 and Phase V. All birds at De Wildt were habituated to captivity and eating regularly. In Phase IV.2 wild *G. africanus* (*n*=25) were captured using a walk-in-trap located at a feeding site for vultures in Namibia [37], run by the Rare and Endangered Species Trust. Captive *G. bengalensis* and *G. indicus* for Phase VI of the trials were held at the Bombay Natural History Society/Haryana State Vulture Conservation Breeding Centre, Pinjore, Haryana State, India. All birds used in Phase I to VI were adults and sub-adults. Ethical issues relating to the experimental protocols were considered and approved by the Animal Use and Care Committee and the Research Committee of the Faculty of Veterinary Science of the University of Pretoria, the Research Council of the Indian Veterinary Research Institute and the Board of the Bombay Natural History Society.

Housing and Management: Birds used for Phases I-III were transported from De Wildt to the University of Pretoria Biomedical Research Centre (UPBRC) seven days prior to the start of Phases I through III. At UPBRC vultures were housed individually in primate cages (1.2 x 0.87 x 0.78 m) in an environmentally controlled room in which the room temperature (19-22 °C) and light cycle were kept constant and humidity was allowed to vary with that outside (between 19% and 50% humidity). Vultures used for Phase IV.1 and Phase V were kept at De Wildt, either within their normal holding aviaries (IV.1), or within smaller isolation cages (V). Birds captured in Namibia (Phase IV.2) were kept in the walk-in-trap (11 x 5.5 x 5.5 m) [37], which doubled as a holding aviary for the seven-day trial. Birds in India were captured from their flight aviaries six days before the start of the trials. Five birds with pre-existing healed wing or leg injuries were held in three small aviaries (4 x 3 x 2.5 m), the remaining two groups of five birds were kept in two large holding aviaries (15 x 10 x 5 m). The vultures were not fed for 24 hours prior to treatment with meloxicam and for up to four hours afterwards. Thereafter birds were fed according to

their normal feeding regime (200 g of meat daily at De Wildt and 1.0 kg of meat every third or fourth day at Pinjore), with the exception of the wild birds in Namibia, which were free to feed from the remains of an adult donkey (*Equus asinus*) placed in the aviary. All meat was from known sources, which were selected because we were confident that they did not use any NSAIDs on their livestock.

Treatment and study design for oral gavage experiments: Phases I-III followed a randomised, two-treatment group, parallel study design with 24 non-releasable captive *G. africanus*. In each phase (I to III), vultures were randomly allocated to a meloxicam-treated group (n=5) and a control group (n=3). In Phase IV.1, we treated 14 captive vultures (no controls) and in Phase IV.2 we treated 21 wild vultures and there were four control birds (Table 3-1). The vultures used in Phase IV.1 had also been used in Phases I-III. To minimise the chance of any effect of earlier treatment we ensured that the interval between the end of one treatment and the beginning of the next was at least six weeks. To minimise the risk to captive *G. bengalensis* and *G. indicus* in India, Phase VI of the meloxicam testing was staggered. Two injured non-releasable birds were first treated by gavage with 0.5 mg kg^{-1} and one control bird was sham-dosed with sterilised water. After 48 hours no apparent ill-effects of the treatment were observed, so a further three birds were dosed with 0.5 mg kg^{-1}, two injured non-releasable birds were dosed with 2 mg kg^{-1}, and a further two control birds were sham-dosed. After another 48 hours, three more birds were dosed with 2 mg kg^{-1} along with two final control birds. All birds (with the exception of birds fed muscle and liver tissue in Phase V) were administered meloxicam as a single dose by oral gavage, with the gavage tube flushed with 2ml of water. Control birds were sham-treated by gavage with sterilised water. Birds were observed following dosing for any regurgitation, but none occurred. The meloxicam used came from >20 bottles of the product purchased from several pharmacies in India. Meloxicam used in all phases of the study was “Melonex”, manufactured by Intas Pharmaceuticals Ltd, Ahmedebad, India. The stated concentration of meloxicam (500 mg l^{-1}) within two bottles was verified against pure meloxicam sodium salt (M-3935, Sigma-Aldrich, St Louis, MO, USA), through the HPLC analysis method described below and found to be within the accepted 10% limits for pharmaceutical products (450 mg l^{-1} and 460 mg l^{-1}).

Phase V treatment and design: Phase V used a randomised three period, three treatment crossover design with a washout period of two weeks between repeat dosing.

Pharamacokinetic studies indicate that meloxicam is rapidly metabolised in five other bird species (elimination half-life ($t_{½\ el}$) of 0.5 to 2.4 hours [20]) and eliminated within 12 hours in *G. africanus* and the two-week washout period was chosen to ensure that no meloxicam residues were likely to be present on repeat dosing. It was intended that each bird should receive all three treatments in turn with a two-week washout period between treatments. The three treatments were (1) feeding with muscle from a meloxicam-treated cow, (2) feeding with liver from a meloxicam-treated cow and (3) oral gavage with a dose of meloxicam intended to be similar to that taken in treatment (2). In each of the three treatment periods, all three treatments were administered to two birds. Hence, two birds were allotted at random to receive the sequence 1,2,3, two to receive 2,3,1 and two to receive 3,1,2. In each treatment period the muscle and liver was taken from one cow. In practice, an error was made so that two birds received the wrong treatment in the final period and instead received 2,3,2 and 3,1,1. Hence, although all three treatments were each administered on six occasions, and to two birds in each of the three periods, two birds received the same type of treatment in two periods. All six birds had previously been trained to consume food from bowls. On the day of dosing two birds were presented with 1 kg of muscle, two birds with 1kg of liver tissue and two birds were dosed by oral gavage. Any food remaining after 48hours was removed and weighed. Doses of meloxicam per kg vulture bw were estimated from the mass of tissue consumed and the concentration of meloxicam within cattle tissues (see below). In the first part of this experiment, neither of the two birds given liver ate much of it, so all six birds were routinely fed liver (between testing sessions) to habituate them to eating liver in the trials.

Treatment of meloxicam dosed cattle for Phase V: Three *Bos taurus* steers of around 18 months of age and weighing 300-400 kg were housed at the UPBRC. Each animal received an intramuscular injection of meloxicam at a dose of 1 mg kg^{-1} on each of five days prior to slaughter. To avoid unnecessary pain the drug volume injected into any one site never exceeded 20 ml, with all injections placed in the neck on the left and right side on alternating days. This dose is twice the lower of the two standard doses (0.5 and 0.7 mg kg^{-1}) recommended for veterinary medicine in India. It is also higher than the dose (0.7 mg kg^{-1}) administered in the EMEA study [22] that we used to calculate the likely MLE of vultures to meloxicam in the wild (Protocol S1). Cattle were slaughtered at the Veterinary Pathology Department, University of Pretoria, by means of captive bolt to the brain

followed by the transection of the spinal cord at the level of the atlanto-occipital junction, without subsequent exsanguination. Each animal was slaughtered eight hours after the last meloxicam dose and on the day prior to vulture feeding. The entire liver and quadriceps femoris muscle were collected (sufficient to supply liver and muscle for two vultures) and refrigerated until feeding on the following day.

Measuring meloxicam in tissues: Meloxicam concentrations in liver and muscle tissues were measured through standard HPLC methods calibrated against a known standard concentration of the drug. Two 1kg pieces of liver and muscle were cut from each slaughtered animal. Five sub-samples of tissue weighing 3-5 g (four from the surface and one from the centre) were taken from each 1 kg block and homogenised. Meloxicam was extracted from a 0.5 g sample of the homogenised tissue, through homogenisation with 2 ml of HPLC grade acetonitrile, which was then centrifuged at 1200 rpm for 10 minutes and subsequently dried at 60 °C under a flow of nitrogen. This was followed up by a clean up process using Waters Oasis (Milford, Ma) HLB solid phase extraction cartridges [38]. The dried eluate was reconstituted in 50 µl MeOH and 100 µl 0.4% acetylacetate and analysed in duplicate by HPLC. For each homogenised sample, the mean of the four values was used as the final estimate of meloxicam concentrations. Meloxicam sodium salt (M-3935, Sigma-Aldrich, St Louis, MO, USA) was used for calibration, with nine standards ranging from 100 to 50,000 µg l^{-1}. The HPLC apparatus comprised a model 126 dual solvent pump, model 168 diode array detector and a 508 autosampler (Beckman Coulter, Fullerton CA, USA). Chromatographic separation was achieved using a Synergi MAX-RP C18 column (2.1 mm x 150 mm, 5 µm; Phenomex, Torrance CA, USA) with UV detection at 275 nm e.g. Quantification was done with peak areas acquired from UV detection at 275 nm.

Observations on vultures: For all birds and all phases, body mass was measured on the day of treatment (day 0) and at the end of each trial period or when birds were returned to their normal aviaries. For Phase I, II, III and V, birds were weighed 12, 8, 12 and 5 days after treatment respectively. Birds from Phase IV.1, IV.2 and VI were weighed on day seven. Body mass was measured to the nearest 0.5 kg (South Africa and Namibia) and 0.1 kg (India). Observations for signs of toxicity and abnormal feeding behaviour were undertaken daily. In Phases I-III, blood (2.5 ml) was taken at 0 h (prior to dosing) and at 4, 12, 24, 48, 96 and 148 h after meloxicam treatment to quantify serum uric acid and albumin concentrations, and creatinine kinase (CK) and ALT activity. In Phase IV blood

(5ml) was taken just prior to dosing and 48 and 168 hours afterwards to determine serum uric acid concentrations. Blood sampling for Phase V was undertaken 24 hours before feeding or dosing by oral gavage, and at 48 and 96 hours after dosing or the start of feeding.

Blood collection from vultures: In Phase I, blood samples were taken by use of an indwelling catheter, placed under anaesthesia in the jugular vein. This procedure was considered to be unsatisfactory, and rapidly abandoned. Subsequently blood samples in all phases of the study were collected by direct veno-puncture from the brachial or tarsal veins. A total of approximately 15 ml of blood (c. 3% of estimated blood volume) was collected from each vulture over a seven-day period.

Measurement of serum constituents: Blood samples were spun at 1200 rpm for 15 minutes in a refrigerated centrifuge (4°C) to separate serum. Uric acid concentration was measured using ACE TM Uric Acid Reagent, albumin concentration using the NExT TM Albumin reagent, ALT activity using the Alfa Wasserman ALT, and CK using the Alfa Wasserman CK Reagent e ACE TM clinical chemistry system (Alfa Wassermann, Bayer Health). The analyses were performed by means of the ACE TM and NExT TM Clinical Chemistry Systems (Alfa Wassermann, Bayer Health Care, SA).

3.6 Acknowledgements

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bengalensis and we are grateful to Chris Bowden for his assistance in procuring permits and helping with the work in India. Jemima Parry-Jones of the National Bird of Prey Conservation Trust and many zoo staff, veterinarians and bird-keepers collaborated with us in the collection of questionnaire data from captive bird collections: these data will be reported in detail elsewhere. This work was funded by the UK Government's Darwin Initiative for the Survival of Species with additional financial support from the Royal Society for the Protection of Birds

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Table 3-1: Summary of results and experimental schedule for the testing of the NSAIDs diclofenac and meloxicam on *Gyps bengalensis* and *G. indicus* vultures, and on the non-threatened *G. africanus*. There was no mortality in any of the control birds.

Gyps species	NSAID	Phase	Dose mg kg^{-1}	Route	*N* dosed	*N* died	% Mortality	*N* control	Status and source of birds
G. bengalensis	Diclofenac	-	0.007 to 0.940	Fed treated tissue	20	13	65	-	Captive birds (Pakistan) [1]
G. bengalensis	Diclofenac	-	0.25 and 2.5	Gavage	4	3	75	2	Captive birds (Pakistan) [1]
G. africanus	Diclofenac	-	0.8	Gavage	2	2	100	2	Captive birds (South Africa) [2]
G. africanus	Meloxicam	I	0.5	Gavage	5	0	0	3	Captive birds (South Africa)
G. africanus	Meloxicam	II	1.0	Gavage	5	0	0	3	Captive birds (South Africa)
G. africanus	Meloxicam	III	2.0	Gavage	5	0	0	3	Captive birds (South Africa)
G. africanus	Meloxicam	IV.1	2.0	Gavage	14 [3]	0	0	-	Captive birds (South Africa)
G. africanus	Meloxicam	IV.2	2.0	Gavage	21	0	0	4	Wild-caught birds (Namibia)
G. africanus	Meloxicam	V	0.03 to 1.98	Fed treated tissue	6 [4]	0	0	-	Captive birds (South Africa)
G. africanus	Meloxicam	V	1.18 to 2.45	Gavage	6 [4]	0	0	-	Captive birds (South Africa)
G. bengalensis	Meloxicam	VI	0.5	Gavage	3	0	0	1	Captive birds (India)
G. bengalensis	Meloxicam	VI	2.0	Gavage	3	0	0	1	Captive birds (India)
G. indicus	Meloxicam	VI	0.5	Gavage	2	0	0	2	Captive birds (India)
G. indicus	Meloxicam	VI	2.0	Gavage	2	0	0	1	Captive birds (India)

[1] Experimental results from reference [1]

[2] Experimental results from reference [7]

[3] Experimental and control birds from phases I to III (including 3 control birds not previously dosed with meloxicam)

[4] 5 of the 6 birds were experimental birds from Phase III and IV.1. The same birds were used for feeding tissue and oral gavage, with a two week washout period between treatments (see Materials and Methods)

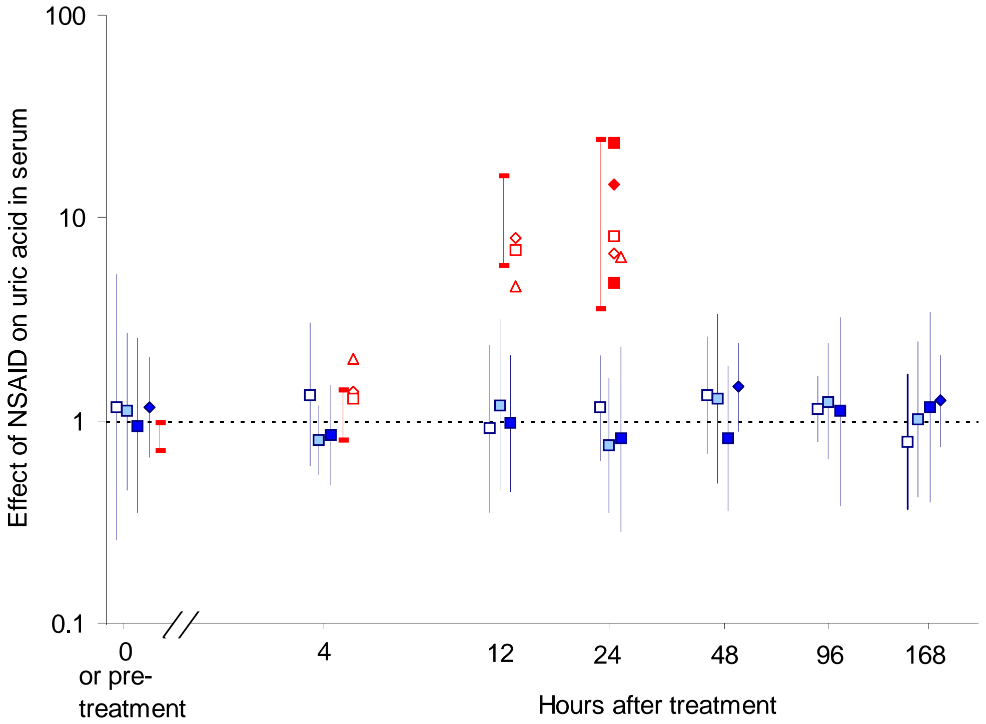
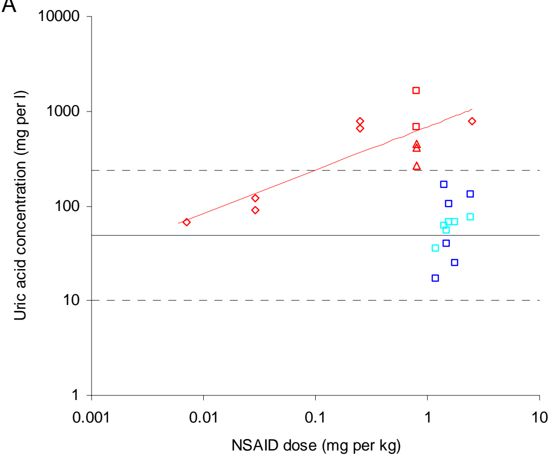
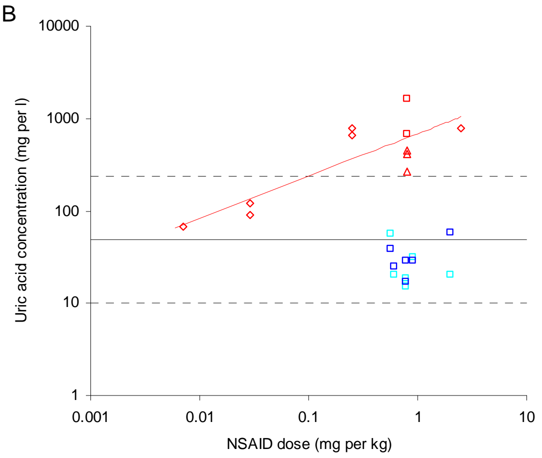


Figure 3-1: Effect of Administration of Meloxicam and Diclofenac by Gavage on Uric Acid in the Serum of Vultures

Blue symbols show the ratio of the geometric mean plasma concentration of uric acid for a group of *Gyps africanus* treated with meloxicam by gavage to that for a control group treated with water and sampled at the same time. Vertical lines show 95% confidence limits for the ratio. The dashed horizontal line indicates a ratio of 1; i.e. no effect of treatment. At each of six times of sampling after treatment, results are shown for experiments in which different doses of drug were used. The fill colour of the blue symbols indicates the meloxicam dose for the treated group: white = 0.5 mg kg^{-1} (Phase I); light blue = 1.0 mg kg^{-1} (Phase II); dark blue = 2.0 mg kg^{-1} (squares = Phase III, diamonds = Phase IV-2). Red vertical bars show the maximum and minimum values of the equivalent ratio for two groups of *G. africanus*, one group treated with 0.8 mg kg^{-1} of diclofenac by gavage and another group treated with water and sampled at the same time. Open red symbols show the ratio of the plasma concentration after treatment to that at the time of treatment for three individual *G. fulvus* given 0.8 mg kg^{-1} of diclofenac by gavage. Filled red symbols show the ratio of the plasma concentration 24 hours post-treatment to that 1 hour post-treatment for three individual *G. bengalensis* given 0.25 mg kg^{-1} (squares) and 2.5 mg kg^{-1} (diamond) of diclofenac by gavage. Data from diclofenac experiments were taken from references 1 and 7.





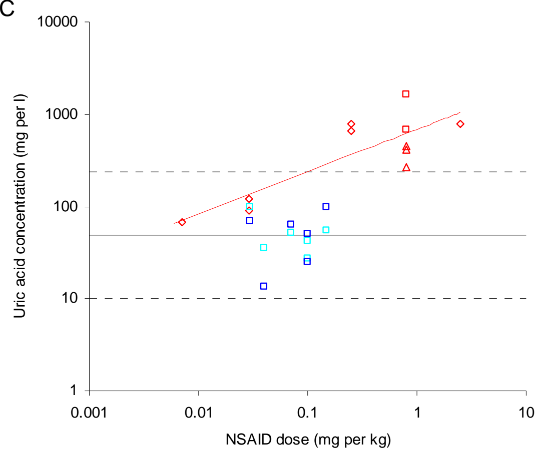


Figure 3-2: Relationship between Uric Acid in Serum the Dose of Meloxicam and Diclofenac Administered and Administration Method

Serum concentration of uric acid in *Gyps africanus* 48 hours (turquoise) and 96 hours (blue) after treatment, in relation to the dose of meloxicam administered per kg of vulture body weight. For comparison, the geometric mean uric acid level (horizontal solid line) and 95% range (horizontal dashed lines) of the experimental birds 24 hours before treatment are shown. Also shown are serum concentrations of uric acid 24 hours after treatment in *G. africanus* (red squares), *G. bengalensis* (red diamonds) and *G. fulvus* (red triangles) to which diclofenac was administered by various methods. The red line shows the regression model fitted to these data. Panels show results for different methods of administration of meloxicam to *G. africanus*: (A) gavage, (B) by feeding liver from meloxicam-treated cattle, (C) by feeding muscle from meloxicam-treated cattle. Data from diclofenac experiments were taken from references 1 and 7.

3.8 Electronic Addendum

3.8.1 *Protocol S2*

Analysis of Phase V data

In addition to the regression analyses reported in the main text, we also examined the Phase V data for subtle effects of feeding *G. africanus* with liver or muscle from meloxicam-treated cattle and dosing with meloxicam by gavage. We carried out analyses of covariance with the log of serum uric acid concentration as the dependent variable. We fitted regression models by ordinary least squares. Independent variables included in the full model were bird identity (BIRD a 6-level factor: 6 birds), stage of the experimental sequence (SEQ a 3-level factor: first, second or third set of trials), time of blood sampling (T a 2-level factor: 48 h or 96 h after treatment began), route by which meloxicam was administered (TREAT a 3-level factor: by feeding liver, by feeding muscle, by gavage), log dose of meloxicam administered (LDOSE a continuous variable) and the log uric acid concentration in the serum each bird before meloxicam was administered in a given trial (LURP a continuous variable). We tested the effect of meloxicam treatment by comparing the following models: (A) BIRD + SEQ + T + LURP + TREAT + LDOSE + TREAT.LDOSE and (B) BIRD + SEQ + T + LURP. This comparison indicated that there was no significant effect of the route of meloxicam dosing and meloxicam dose on serum uric acid level ($F_{5,21} = 0.58$, $p > 0.50$). This was also the case when the equivalent analysis was performed separately for data collected 48 h after dosing and 96 h after dosing and when the tissue feeding and gavage routes of administration were subject to two separate analyses. We also fitted models to the full dataset with all possible combinations of the variables listed above, including the TREAT.LDOSE interaction. We then used the results to select the Minimal Adequate Model (MAM; Crawley 1993). The MAM was BIRD + LURP. In this model, both of these two variables had highly significant effects (BIRD: $F_{5,29} = 5.34$, $p < 0.005$; LURP: $F_{1,29} = 17.26$, $p < 0.001$), but no other variable or combination of variables had a significant further effect ($p > 0.10$) when added to the MAM.

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Table 3-2: Blood serum constituents summary statistics

Results of analyses of measurements of uric acid and albumin concentrations, and alanine transferase (ALT) and creatinine kinase (CK) activity in blood serum before dosing and at 0, 4, 12, 23, 48, 96 and 168 hours after dosing for birds from Phase I (meloxicam at 0.5 mg kg^{-1}), Phase II (1.0 mg kg^{-1}) and Phase III and Phase IV (2.0 mg kg^{-1}). Blood was only collected at 0, 48 and 168 hours in Phase IV. The geometric mean of meloxicam-dosed and control (sham-treated) birds is presented along with the sample size and *P* values from two-tailed t-tests between meloxicam-dosed and control groups.

		Uric acid mg l^{-1}			Albumin mg l^{-1}			ALT U l^{-1} (37° C)			CK U l^{-1} (37° C)		
Phase	Time h	Control Mean (n)	Dosed Mean (n)	*p*	Control Mean (n)	Dosed Mean (n)	*P*	Control Mean (n)	Dosed Mean (n)	*p*	Control Mean (n)	Dosed Mean (n)	*p*
Phase I dose 0.5 mg kg^{-1}	0	34.5 (3)	40.2 (5)	> 0.80	11.3 (3)	13.1 (5)	> 0.10	21.0 (3)	33.1 (5)	> 0.10	432.2 (3)	235.1 (5)	> 0.05
	4	23.9 (3)	32.1 (5)	> 0.40	11.3 (3)	13.8 (5)	> 0.20	45.7 (3)	38.4 (5)	> 0.70	591.1 (3)	459.7 (5)	> 0.30
	12	116.9 (3)	106.5 (5)	> 0.80	11.7 (3)	13.3 (5)	> 0.30	31.9 (3)	31.0 (5)	> 0.90	506.7 (3)	472.6 (5)	> 0.80
	24	44.7 (3)	51.7 (5)	> 0.50	12.2 (3)	14.2 (5)	> 0.20	43.0 (3)	40.0 (5)	> 0.80	440.1 (3)	443.5 (5)	> 0.95
	48	48.7 (2)	64.8 (4)	> 0.30	13.1 (2)	15.2 (4)	> 0.20	69.8 (2)	39.5 (4)	> 0.10	386.4 (2)	347.1 (4)	> 0.80
	96	55.6 (3)	63.2 (5)	> 0.40	11.8 (3)	13.9 (5)	> 0.05	51.3 (3)	43.3 (5)	> 0.05	461.7 (3)	603.2 (5)	> 0.30
	168	99.8 (3)	79.1 (5)	> 0.40	12.5 (3)	14.0 (5)	> 0.10	51.3 (3)	46.0 (5)	> 0.50	570.6 (3)	539.0 (5)	> 0.80
Phase II dose 1.0 mg kg^{-1}	0	72.0 (3)	80.2 (5)	> 0.70	11.1 (3)	11.5 (5)	> 0.50	32.5 (3)	19.0 (5)	> 0.20	178.0 (3)	223.9 (5)	> 0.20
	4	39.8 (3)	32.0 (5)	> 0.20	12.1 (3)	11.3 (5)	> 0.40	28.5 (3)	23.1 (5)	> 0.50	442.2 (3)	603.2 (5)	> 0.50
	12	125.8 (3)	150.4 (5)	> 0.60	17.7 (3)	11.1 (5)	> 0.20	72.4 (3)	37.5 (5)	> 0.30	842.4 (3)	397.2 (5)	> 0.40
	24	73.9 (3)	55.6 (5)	> 0.30	10.1 (3)	10.9 (5)	> 0.20	33.8 (3)	44.4 (5)	> 0.20	327.8 (3)	492.8 (5)	> 0.30
	48	73.0 (3)	93.5 (5)	> 0.50	10.4 (3)	11.2 (5)	> 0.10	43.0 (3)	44.9 (5)	> 0.80	278.7 (3)	405.2 (5)	> 0.30
	96	124.6 (3)	154.5 (5)	> 0.40	11.8 (3)	12.9 (5)	> 0.20	39.8 (3)	42.5 (5)	> 0.70	316.4 (3)	234.6 (5)	> 0.30
	168	114.5 (3)	116.3 (5)	> 0.95	10.6 (3)	11.6 (5)	> 0.05	54.9 (3)	50.8 (5)	> 0.60	327.1 (3)	327.2 (5)	> 0.95
Phase III dose 2.0 mg kg^{-1}	0	60.1 (3)	56.8 (5)	> 0.80	12.6 (3)	11.6 (5)	> 0.05	76.6 (3)	74.0 (5)	> 0.70	243.0 (3)	318.1 (5)	> 0.40
	4	21.2 (3)	18.1 (5)	> 0.50	11.8 (3)	10.6 (5)	> 0.10	37.2 (3)	46.5 (5)	> 0.50	431.4 (3)	459.3 (5)	> 0.70
	12	96.3 (3)	93.1 (5)	> 0.90	11.0 (3)	10.0 (5)	> 0.10	36.6 (3)	40.7 (5)	> 0.70	328.3 (3)	506.9 (5)	> 0.05
	24	63.8 (3)	51.8 (5)	> 0.60	10.8 (3)	10.1 (5)	> 0.40	40.6 (3)	46.8 (5)	> 0.40	278.7 (3)	438.9 (5)	> 0.10
	48	65.4 (3)	53.2 (5)	> 0.50	10.5 (3)	9.7 (5)	> 0.30	47.9 (3)	40.4 (5)	> 0.60	220.4 (3)	357.6 (5)	> 0.10
	96	76.5 (3)	84.7 (5)	> 0.80	11.5 (3)	10.2 (5)	> 0.10	55.2 (3)	52.7 (5)	> 0.90	236.1 (3)	429.3 (5)	> 0.20
	168	73.3 (3)	84.8 (5)	> 0.70	12.8 (3)	11.7 (5)	> 0.20	44.3 (3)	54.2 (5)	> 0.60	531.7 (3)	647.2 (5)	> 0.60
Phase IV dose 2.0 mg kg^{-1}	0	95.4 (4)	111.7 (21)	> 0.50	-	-	-	-	-	-	-	-	-
	48	70.5 (4)	102.9 (21)	> 0.10	-	-	-	-	-	-	-	-	-
	168	100.2 (4)	125.4 (21)	> 0.30	-	-	-	-	-	-	-	-	-

CHAPTER 4: The pharmacokinetics of meloxicam in vultures

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The pharmacokinetics of meloxicam in vultures

V Naidoo[3], K Wolter[2], A D Cromarty[3], P Bartels[4], L Bekker[1], L McGaw[1], M A Taggart[5,6,7], R Cuthbert[6], GE Swan[1]

[1]Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, South Africa

[2]Vulture Unit, De Wildt Cheetah and Wildlife Trust, South Africa

[3]Department of Pharmacology, Faculty of Health Sciences, University of Pretoria, South Africa

[4] Wildlife Biodiversity Resources, National Zoological Gardens of SA, Pretoria, South Africa

[5]School of Biological Sciences, Department of Plant and Soil Science, University of Aberdeen, St Machar Drive, Aberdeen, AB24 3UU, Scotland, UK.

[6]Royal Society for the Protection of Birds, The Lodge, Sandy, Bedfordshire, SG19 2DL, England, UK.

[7]Instituto de Investigación en Recursos Cinegéticos, Ronda de Toledo s/n, 13005, Ciudad Real, Spain,

4.1 Abstract

Vulture populations across the Asian subcontinent have declined dramatically in the last fifteen years and are now on the verge of extinction. Although the cause of the population decline was initially unknown, the decrease has recently been conclusively linked to the use of the non-steroidal anti-inflammatory drug diclofenac in cattle that inadvertently ended up in the vulture food chain. With the vulture numbers continuing to decline by up to 48% a year, the Indian, Nepali and Pakistan governments have recently banned the manufacture and importation of veterinary diclofenac. They have also suggested meloxicam as an alternate anti-inflammatory for use in cattle. This recommendation was based on extensive acute safety studies in the African White-backed Vulture (*Gyps africanus*) which evaluated worst case scenarios of maximum intake based on a once in

[3] Private Bag X04, Section of Pharmacology and Toxicology, Faculty of Veterinary Science, Onderstepoort 0110, South Africa. Vinny.naidoo@up.ac.za

three day feeding pattern. However, the possible cumulative pharmacokinetic and pharmacodynamic effects in vultures receiving multiple daily doses of meloxicam over time were not assessed. At present very little pharmacokinetic or pharmacodynamic information is available to add further support for the safety of meloxicam in this animal species. This article discusses the oral and intramuscular pharmacokinetics of meloxicam in Cape Griffon vultures (*Gyps coprotheres*). Therapeutic drug monitoring was also undertaken in White-backed, Egyptian (*Neophron pernopterus*) and one Lappet Faced Vulture (*Torgos tracheliotos*). In all these species meloxicam was characterised by a short half-life of elimination. The rapid metabolism of meloxicam in combination with a short duration of effect in the studied *Gyps* vulture species shown in this study makes it unlikely that the drug could accumulate. This confirms the safety of repeated exposure to meloxicam in vultures of this genus.

Keywords: Meloxicam, Vultures, Safety, Diclofenac, NSAID, COX

4.2 Introduction

Three species of vultures endemic to South Asia are in grave danger of extinction across the Indian subcontinent. Populations of Oriental white-backed vultures (*Gyps bengalensis*), long-billed vultures (*G. indicus*) and slender-billed vultures (*G. tenuirostris*) have declined by more than 97% in India and Pakistan (Shultz *et al.*., 2004) and continue to decline at rates of 22-48% per year (Green *et al.*., 2004). In 2004, Oaks *et al.*., linked the population crash to the veterinary use of diclofenac, a non-steroidal anti-inflammatory drug (NSAID). In this study, Oaks *et al.*. (2004) was able to demonstrate that diclofenac residues in the carcasses of dead cattle, treated prior to their deaths, were highly toxic to scavenging vultures. From subsequent modelling data Green *et al.* (2004) were able to show that residues in few as one in approximately two hundred carcasses would be sufficient to cause the decline rates reported. Diclofenac has also been shown to have an approximate LD_{50} of 0.098 to 0.225 mg/kg in vultures (Swan *et al.*., 2006b), making it more lethal than aldicarb, which is regarded as one of the most toxic pharmaceutical compound in animals (Pesticide Information Profiles, 1996).

To protect the remaining vulture populations, the governments of India, Pakistan and Nepal have taken steps to phase out the veterinary use of diclofenac, including bans on the manufacture and importation of the drug in addition to recommending the use of vulture safe alternatives such as meloxicam for livestock treatment (Mo, 2006). The recommendation to use meloxicam was based on an extensive safety-testing study, in which *Gyps* vultures were exposed to oral meloxicam as either pure drug in formulation or residues in tissues from livestock dosed with meloxicam (Swan *et al*.., 2006a; Swarup *et al*.. 2007). While these studies have demonstrated the safety of meloxicam (in comparison to diclofenac) following single exposure to the drug, the safety of meloxicam following repeated exposure was not documented. Swan *et al*.. (2006a) estimated the maximum levels of exposure on the basis of meloxicam residues in livestock liver tissues shortly after dosing and for a vulture consuming enough liver tissue at one sitting (1.02kg) to provide the estimated energetic requirements for three days: a plausible maximum duration between meals based on observations of wild and captive vultures (Mundy *et al*.., 1994). This does not, however, consider the cumulative effect of multiple exposures to meloxicam over a short time period, which may occur for birds consuming livestock tissues on a daily basis.

Irrespective of the duration of exposure, when evaluating the safety of a NSAID, one other factor which must be considered is the duration of cyclo-oxygenase (COX) enzyme inhibition i.e. should COX inhibition be reversible, the time required for complete enzyme recovery is important (Boothe, 2001). As an illustration, carprofen is effective for once a day pain management in the dog despite the drug having a half-life of just 8 hours (Clark, 2006). As such it is plausible that a drug with a short pharmacokinetic half-life (non-cumulative) could still result in toxicity if successive doses lead to prolonged enzyme inhibition mechanistically.

In this study we characterise the intramuscular and oral bioavailability of a single dose of meloxicam in adult *G. corportheres* vultures to determine the elimination half-life of the drug and if possible to extrapolate the plasma profile for multiple exposures over time. The safety of meloxicam was also evaluated by monitoring birds treated on a daily basis.

4.3 Material and Methods

4.3.1 *Pharmacokinetic Study*

The pharmacokinetics (PK) of meloxicam in adult Cape Griffon vultures was evaluated using a single dose, two phase parallel study consisting of six birds per treatment group (Table 4-1). The birds were allowed an acclimatization period of one week. To facilitate the management of the study the intramuscular (i.m.) and oral dosing was separated by one week. The vultures were housed within the University of Pretoria's Biomedical Research Centre (UPBRC) in single aviaries of 1x1x2.5 metres. During the study the birds were fed twice weekly with one kilogram of beef each, bought from a commercial butchery. The meat was assumed to be free of meloxicam and other NSAIDs as South Africa follows the minimum residue limit guidelines proposed by the joint expert committee on food additives of the FAO and WHO (JECFA) in the determination of withdrawal periods (Act 54/72). At the end of the study, the birds were returned to De Wildt Cheetah and Wildlife Centre from where they were sourced. All captive animals used in this study were in captivity for at least one year prior to inclusion in the study.

The birds were given a single dose of meloxicam (Melonex 0.5%m/v, India) at 2 mg/kg by either intramuscular injection or oral gavage. Dose rates of 2 mg/kg were selected, as this is the estimated maximum level of exposure to meloxicam used in previous safety testing (Swan *et al.*., 2006). For gavage a small diameter tube was passed directly into the crop. Once the drug was dosed, the tube was flushed with 2 ml of sterile water. Once the tube was removed, a further 2 ml of water was squirted into the mouth of the vulture. Intramuscular injections were administered directly into the pectoral muscle.

Blood samples were collected by means of a 5ml syringe and immediately transferred into 5ml lithium heparinised vacutainer. Samples were collected generally from the tarsal vein or when necessary the wing vein, before drug administration and at 4 and 30 minutes and at 1, 1.5, 2, 6, 8 and 10 hrs after treatment. Within two hours of collection the blood samples were centrifuged at ca 3000*g* and 4°C for 15 min and the supernatant of each sample transferred to labelled polycarbonate tubes.

Plasma concentration data for all animals were analysed using WinNonLin 4.2 (sponsored by the Pharsight Corporation). The plasma curve for meloxicam for both intramuscular and oral routes were best fitted to a one compartment open model (model 3) and were best described by the equation 1:

$$C = \frac{\text{Dose} \times K_a}{V_d/F \times (K_a - K_e)} \times (e^{-kat} - e^{-ket}) \qquad (1)$$

where C is the plasma concentration at time t, K_a the absorption constant, K_e the elimination constant and V_d/F the apparent volume of distribution. The relative bioavailability ($F_{relative}$) was calculated according to equation 2.

$$F_{relative} = \frac{AUC_{oral}}{AUC_{im}} \times 100 \qquad (2)$$

where AUC represents the area under curve to the last time point for the oral and i.m. routes of administration.

4.3.2 *Liquid chromatography tandem mass spectrometry*

Sample extraction and preparation was done using a method developed in our laboratory and reported previously (Swan *et al.*, 2006a). Briefly, 2 ml acetonitrile was added to 200 µl of plasma, mixed for 4 min on a Lab-tek multitube vortexer and subsequently centrifuged at 1200*g*. The supernatants were transferred to clean glass tubes and evaporated to dryness at 60 °C under a stream of nitrogen gas in a Zymak TurboVap® LV Evaporator. Reconstitution was performed with 50 µl 100% methanol, followed by addition of 100 µl 0.4% acetic acid in methanol/dH_2O (60:40).

The sample extracts were analysed by LC/MS/MS using an Agilent 1100 series high pressure liquid chromatograph with temperature controlled autosampler and diode array detector (collecting the cumulative absorbance from 210 to 400nm) coupled to an Applied Biosystems API4000 QTrap mass spectrometer fitted with a "Turbo V" electrospray ionisation (ESI) source. The HPLC column used was a Phenomenex Prodigy ODS(3) C18 column (4.6x100 mm, 3 µm particle size) and the mobile phase a 20:80 mixture of A: 0.1% formic acid and B: 60% acetonitrile in 0.1% formic acid at a flow rate of 1000 µl/min for 6.5 minutes (Wiesner *et al.*, 2003). The sample injection volume used was 2 µl. The ion

source was operated in the positive mode at 450˚C with the source-specific nebuliser and source gasses set at the optimal pressures as determined during FIA optimisation. Analytes were detected and quantitated by means of characteristic ion transitions from protonated parent ions to fragment ions generated by collisionally activated dissociation (CAD) utilizing the multiple reaction monitoring mode (MRM). The collision gas was nitrogen at the high setting (using a CMC nitrogen generator), and collision energies were optimised for each analyte as listed in Table 2. Additionally the extracted wavelength diode array chromatograms (350±20nm) were used to confirm the retention times of the meloxicam metabolites. The method was shown to have no interference when plasma from untreated birds were injected and demonstrated a limit of detection (LOD), defined at a signal-to-noise ratio 3:1, was less than 30 ng/ml for meloxicam while the limit of quantitation (LOQ) was less than 125 ng/ml in spiked plasma. Calibration curves for meloxicam were linear over a range of 125 – 12500 ng/ml with regression coefficients of at least 0.99. Average accuracy over the concentration range analysed was 96% and precision varied from 13% to 0.5% depending on the concentration with the highest variation observed at the lowest concentrations.

Studies on metabolite ion fragmentation were conducted by direct infusion of plasma extracts, known to contain the metabolites, dissolved in 30% acetonitrile and 0.1% formic acid, into the ESI source. In order to elucidate the origin of the fragment ions and potential dissociation pathways, MS3 experiments were performed on samples containing the metabolites (Table 4-2).

4.3.3 *Meloxicam Clinical and Therapeutic Monitoring*

Some of the birds included in this study were injured (soft tissue injuries) and showed signs of pain such as drooping heads, decreased feed intake and reluctance to place weight on their injured limb or fly (Table 1). In total 11 vultures from four difference species were monitored for signs of toxicity following treatment with meloxicam at the dose of 2mg/kg, by intramuscular administration into the pectoral muscle. With the exception of the White-backed vultures, which received only one dose of meloxicam, all the birds received multiple treatments at 24h intervals.

Of the treated vultures one Cape vulture (*G. coprotheres*) and White-backed vulture (*G. africanus*) were monitored for clinical signs of toxicity for their 14 and 5 days of treatment respectively, without quantifying plasma concentrations. For the other birds, prior to each 24 hour treatment, plasma samples were collected and analysed for meloxicam concentrations as described above. This included one Lappet Faced vulture (*T. tracheliotos*) from which samples were collected at 0, 24, 48, 72, 96 and 120 hours; one Egyptian vulture (*N. pernopterus*) from which samples were collected at 0, 4, 18 and 24 hours and five African White-backed Vultures (*G. africanus*) from which samples were collected at 0, 4, 12, 24 hours.

4.4 Results

4.4.1 *Pharmacokinetics*

The pharmacokinetic parameters obtained are listed in Table 4-3 for both routes and illustrated in Figure 4-1. Oral absorption of meloxicam in the vultures was characterised by a relative bioavailability of 107% compared to intramuscular absorption. Meloxicam was also rapidly absorbed with C_{max} being achieved within approximately 0.5 hours of administration for both routes of administration. The absorption half life ($T_{1/2\alpha}$) of 0.41 ± 0.33h and 0.33 ± 0.17h for the intramuscular and oral routes respectively was very similar to their elimination half life ($T_{1/2\beta}$) of 0.42 ± 0.1h and 0.32 ± 0.17h respectively. A small apparent volume of distribution (V_d/F) was observed following both oral and intramuscular administration.

4.4.2 *Biotransformation Pathways*

We identified three peaks as potential metabolites due to their time dependent increase in concentration in conjunction with meloxicam's decline (Figure 4-2). Based on molecular mass they correspond to two hydroxy-methyl-metabolites together with one glucuronide conjugate (Table 4-2).

4.4.3 *Meloxicam Clinical and Therapeutic Monitoring*

No signs of toxicity were seen in the Egyptian (*N. pernopterus*), Lappet Faced (*T. tracheliotos*), African White-backed (*G. africanus*) or Cape Griffon (*G. corprotheres*) vultures following repeated treatment with parenteral meloxicam. While no drug was detectable for the Lappet Faced vulture, the plasma concentrations in the Egyptian vulture was 5 µg/ml at 4 hours. Meloxicam (0.22 ± 0.20µg/ml) was detectable for all the dosed White-backed vultures in which a 4 hour sample was collected. None of the other time points, had detectable drug concentrations.

The Cape Griffon Vulture that was treated with meloxicam for 14 days was eventually terminated due to non-recovery for an injured leg. From the full post-mortem examination of this bird, no gross pathological lesions were evident, except muscle atrophy for the injured limb.

4.5 Discussion

4.5.1 *Pharmacokinetics*

Although the reason for the higher oral bioavailability (107%) relative to the intramuscular route is unknown it may be an artefact as absorption was more rapid following oral administration resulting in the increased oral C_{max} . In our opinion this is the reason for the higher AUC_{oral} and is a reflection of the sampling intervals rather than drug effect. This supported by Toutain *et al.* (2004), who stated that small differences of this nature are usually non-significant and may result from the predetermined sampling interval. However, to determine the actual significance of the higher oral bioavailability the absolute bioavailability for both routes will have to be determined.

With the absorption and elimination half-lives being almost identical for each route, this tends to suggest that absorption is the limiting factor in the rate of elimination of the drug. It is therefore possible that a degree of flip-flop kinetics is being seen and requires further investigation following intravenous administration. A V_d/F was observed following both oral and intramuscular administration. The result was similar to results previously reported

for the pigeon and a consistent finding for most NSAIDs (Boothe, 2001; Baert, 2003). With the NSAIDs being highly plasma protein bound in birds, the low V_d/F likely results from extensive macro-molecular binding and possibly rapid metabolism. (Lees *et al.*., 2004; Boothe, 2001; Baert *et al.*., 2002; Baert *et al.*., 2003).

This rapid half-life of elimination is extremely important as it can prevent drug accumulation and delayed toxicity. With the assumption that 99% of all drug is eliminated in ten elimination half-lives, these birds will be virtually free of the drug 5-7 hours post exposure (Brown, 2001). The main objective of this study was to assess the potential for meloxicam's accumulation following multiple feedings: given the observed time to elimination a vulture will have to consume numerous meals in one day for accumulation to occur. Ecological studies of vultures in the wild indicate that it is normal for birds to engorge themselves at one session if sufficient food is available, making it unlikely that a bird will be able to consume more than one meal a day (Mundy *et al.*., 1992). Moreover, the dose of 2.0 mg/kg administered in this study, is the likely maximum level of exposure based upon a bird consuming three days of food (1.02 kg) at just one sitting, and consuming only liver tissues from an animal dosed with meloxicam in the hours immediately prior to death. Given this scenario, it is very unlikely that birds can take in larger concentrations of meloxicam at levels able to cause accumulation.

With this study making use of the drug in formulation instead of residues in meat, it may be possible that the presence of meat could adversely influence the process of absorption. i.e. food decreasing the rate of absorption. Although this has never been specifically found with meloxicam, the consumption of food in people is known to slow the rate of absorption of ketoprofen (Busch *et al.*, 1990; Bannwarth *et al.*, 2004). Unfortunately we are doubtful that the influence of a meal on meloxicam's absorption can ever be properly determined in the vulture as unlike mammals, one of the defence mechanisms of the vulture is to regurgitate its meal at the first sign of a threat (Mundey *et al.*, 1992). This is believed to have two important effects, firstly to scare off the threatening animal and secondly to make the body lighter and quicken escape times. As a result it is impossible to get close to these birds soon after feeding for blood collection, as they immediately regurgitate the ingested meal. Realistically we don't believe that will have a major influence on the safety of the

product as slower absorption should promote lower plasma concentrations and lower the drug's toxic potential.

4.5.2 *Biotransformation Pathway*

Generally in mammals meloxicam is metabolised by CYP2C9 during the Phase I reactions and by glucuronide transferase in PhaseII. The predominant Phase I metabolites in laboratory animals and man are the 5-hydroxymethyl derivative and a 5-carboxy metabolite (Busch, 1998; Chesne, 1998). With two hydroxy-methyl-metabolites together with one glucuronide conjugate being tentatively identified, it is likely that vultures make use of the cytochrome P450 enzyme system, perhaps even CYP2C9 as in man, for initial metabolic transformation and glucuronidation for the synthetic reaction (Chesne *et al..*, 1998). The presence of a glucuronide conjugate also indicates that this species appears to use the standard metabolic pathways for NSAID metabolism as described in other animals (Baert and De, 2003; Busch *et al..*, 1998; Kumar *et al..*, 2002). Unlike in mammals the carboxy-metabolite (m/z = 391) was absent. Since the hydroxyl metabolite is converted to a carboxy metabolite by a non-cytochrome dependant pathway, the absence of the peak suggests the absence of these pathways in the vulture (Chesne *et al..*, 1998). More work is, however required to confirm these finding.

4.5.3 *Meloxicam Clinical and Therapeutic Monitoring*

For the Egyptian vulture in which drug was detectable at only 4 hours, we estimate a $T_{1/2\beta}$ of below 2 hours. More birds will have to be evaluated to confirm the half-life of the drug in the Egyptian vulture. As for the Egyptian vulture, the terminal half-life could be established for the White-backed vulture as only one time point had detectable drug concentrations. None the less the plasma concentration for this species fits the equation described for meloxicam disposition in the *G. corprotheres* where the concentration is 0.29 ±0.27 µg/ml at 4h. This tends to suggest that the half-life and absorption of meloxicam is likely to be similar in the two species.

The lack of notable pathology in the Cape vulture treated for two weeks in addition to the absence of clinical signs of toxicity in any of the birds receiving repeated therapy was considered a significant finding as this clearly shows that toxicity will not result from the cumulative exposure to the product.

4.6 Conclusion

The anti-inflammatory drug, meloxicam appears to be rapidly metabolised and excreted in vultures. The rapid excretion in the vulture species tested may indicate that the metabolism is similar in all four of these vulture species. This study therefore, goes some extent towards explaining the safety of the drug in this species and implies that meloxicam is unlikely to have a toxic effect in birds feeding once a day. For vultures being treated therapeutically it is possible that with a rapid half-life birds may require more frequent therapy than the daily regime used at present. Although twice a day dosing may be more helpful, dosage intervals should also be based on the duration of apparent analgesic effect.

4.7 Acknowledgements

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Table 4-1: A list of the different birds included in this study. All the listed birds were in captivity following attempted rehabilitation

Vulture Species	**n**	**Route**	**Status**	**Health Status**	**Analysis**
G. coprotheres	6	Im	Captive	Healthy	Meloxicam PK
G. coprotheres	6	Oral	Captive	Healthy	Meloxicam PK
G. africanus	8	Oral	Captive	Healthy	Meloxicam TDM
N. pernopterus	1	Im	Captive	Injured leg	Meloxicam TDM
G. africanus	1	Im	Wild	Injured Wing	Meloxicam TDM
T. tracheliotos	1	Im	Captive	Injured leg	Meloxicam TDM
G. coprotheres	1	Im	Wild	Injured leg	Monitored for response to meloxicam

Table 4-2: The analytical and mass spectrometer parameters used in identifying the metabolites of meloxicam in *G. corprotheres* plasma samples

Metabolite	MW (Da)	Characteristic transitions (% abundance)			CE (eV)	RT (min)
Meloxicam parent	351.4	352>115 (100)*	352>141 (40)	352>153 (5)	65	5.08
Mono-hydroxy 1	367.4	368>115*			50	3.51
Mono-hydroxy 2	367.4	368>115*			50	2.83
Glucuronide	543	544>115*			65	1.61
Carboxy	381	382>115*			65	ND

* MRM transition signal used for quantitation of the compound.; ND-non detected

Table 4-3: Pharmacokinetics parameters for meloxicam following intramuscular and oral administration in *G. corprotheres* using a one compartmental analysis

Parameter	**Unit**	**IM**		**Oral**	
		Mean (n=6)	**%CV**	**Mean (n=6)**	**%CV**
Ka	h^{-1}	1.77	27.28	2.56	45.10
$T_{1/2\alpha}$	H	0.41	24.14	0.33	53.19
C_{max}	µg/mL	3.58	44.33	5.25	33.51
T_{max}	H	0.60	25.31	0.47	52.78
Ke	h^{-1}	1.75	29.55	2.57	0.01
$T_{1/2\beta}$	H	0.42	26.56	0.32	52.37
AUC	µg/mL*h	5.86	58.50	6.29	41.78
Cl/F	mL/kg/h	130.20	130.79	56.82	62.52
V_d/F	L/kg	0.26	0.00	0.15	0.00

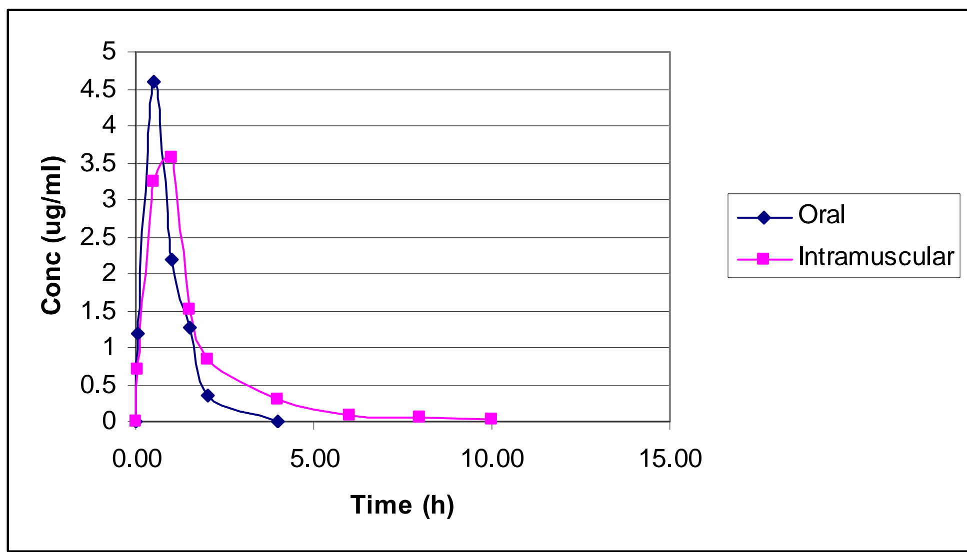

Figure 4-1: Mean plasma concentration versus time curve following oral and intramuscular meloxicam administration in adult *G. corprotheres* vultures.

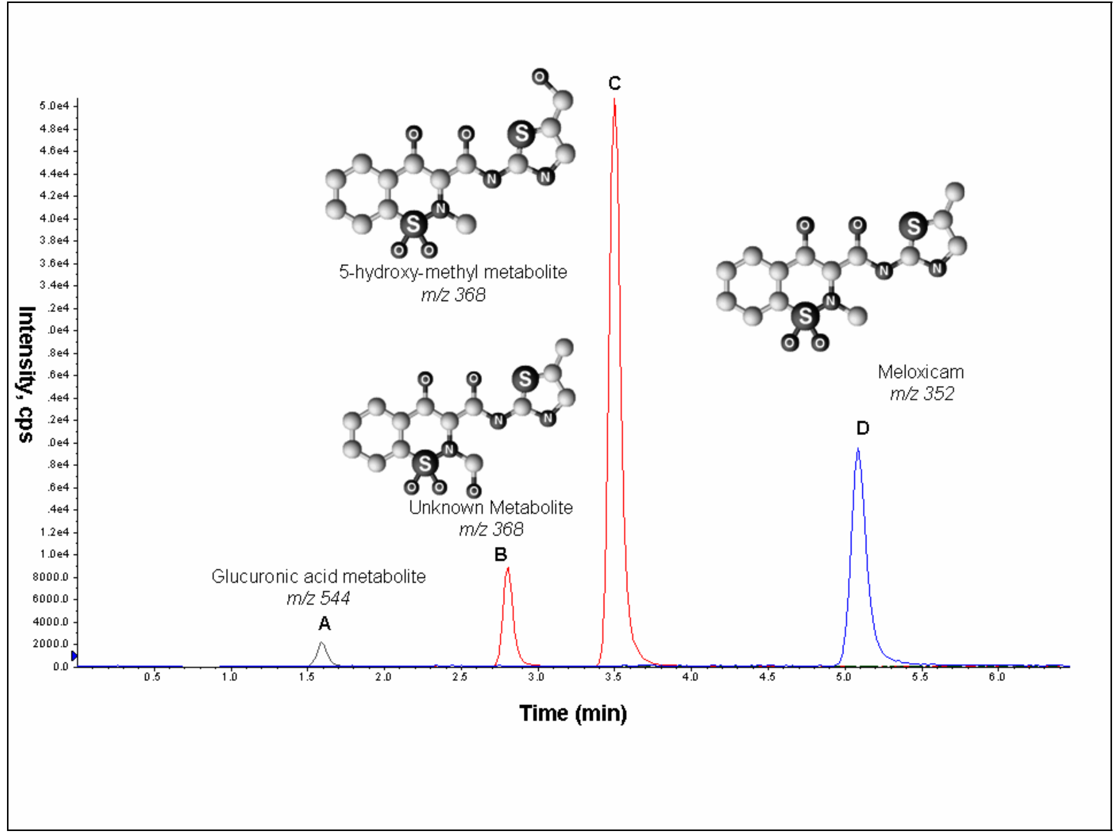

Figure 4-2: Identified meloxicam metabolites as determined by LCMSMS. a) Glucuronide metabolite, b) hydroxyl metabolite 1 c) the unknown metabolite indentified as potential second hydroxyl metabolite, d) meloxicam

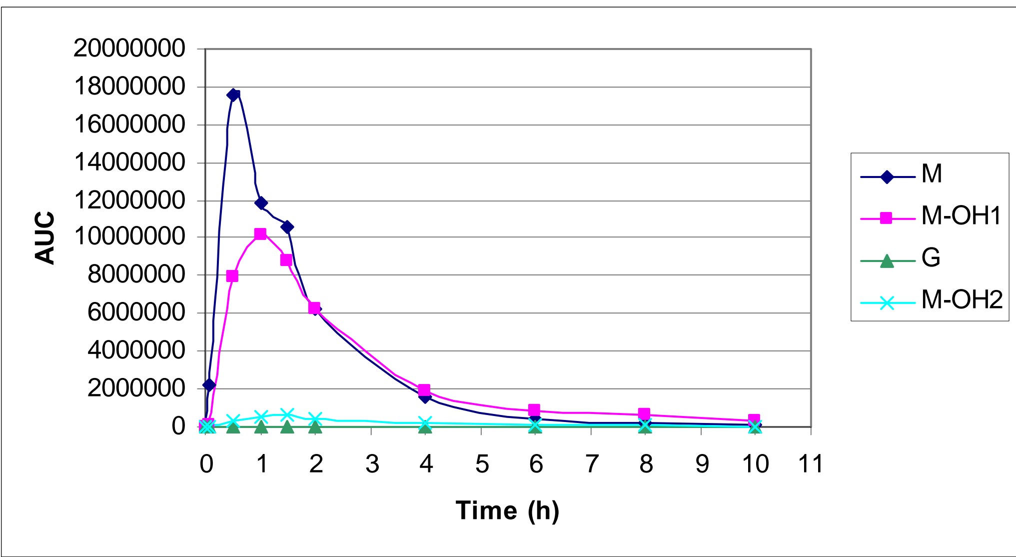

Figure 4-3: Change in the average area under curve over time for each metabolite following the oral administration of meloxicam, using diode-array detection. The initial increase over time corresponded to a decrease in plasma meloxicam concentrations (M: Meloxicam parent, M-OH1- Hydroxy metabolite 1, G-glucuronide metabolite, M-OH2- Hydroxy metabolite 2)

CHAPTER 5: Validating the domestic fowl as a model to investigate the pathophysiology of diclofenac in Gyps vultures

The following manuscript has been published in the Journal of Environmental Pharmacology and Toxicology: Naidoo, V., Duncan, N., Bekker, L. & Swan, G. (2007), Validating the domestic fowl as a model to investigate the pathophysiology of diclofenac in Gyps vultures. *Environmental Toxicology and Pharmacology*, 24, 260-266.

VALIDATING THE DOMESTIC FOWL AS A MODEL TO INVESTIGATE THE PATHOPHYSIOLOGY OF DICLOFENAC IN GYPS VULTURES

V Naidoo[4], N Duncan, L Bekker, G Swan

Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, South Africa

5.1 Abstract

Diclofenac has recently been identified as a cause of the widespread vulture decline on the Indian subcontinent. Although the clinical signs and pathology have been described, the pathophysiology of toxicity remains unexplained. In the following study we attempt to validate the domestic fowl as a model, to allow for the further characterisation of diclofenac's mechanism of toxicity. In a lethal dose study, diclofenac was shown to have an approximate intramuscular LD_{50} of 9.8 mg/kg in 18 week old layers. Signs of toxicity in the affected birds were severe depression that persisted from 24 hours post dosing to death with corresponding increased plasma uric acid concentrations. Post-mortem examinations showed signs of gout with deposits of urates (tophi) in the kidneys, liver, heart and spleen. The pharmacokinetics after both the intramuscular and oral route showed that diclofenac had a short half-life of elimination of approximately one hour, a volume of distribution of 0.09 to 0.24 L/kg and relative oral bioavailability of 50% compared to intramuscular administration. With the similarity in the clinical signs, necropsy findings, histopatological lesions and clinical pathological changes, the fowl may be used in further studies to characterise the mechanism of toxicity of diclofenac. However, due to the large difference in susceptibility of the fowl, it is not a suitable model to simulate the dose-response relationship of the vulture to the other non-steroidal anti-inflammatory drugs.

Key Words: Vulture, *Gyps africanus*, Diclofenac, Pharmacokinetics, LD_{50}, hyperkalaemia

[4] Private Bag X04, Section of Pharmacology and Toxicology, Onderstepoort, 0110, South Africa. +2712 529 8368. vinny.naidoo@up.ac.za

5.2 Introduction

Three species of vultures endemic to South Asia are in grave danger of extinction across the Indian subcontinent. Populations of the Oriental white-backed vulture (*Gyps bengalensis*), long-billed vulture (*G. indicus*) and slender-billed vulture (*G. tenuirostris*) have declined by more than 97% in India and Pakistan, with the result that all three species have now been classified by the IUCN, The World Conservation Union, as Critically Endangered (Prakash *et al.*., 2003). Although the devastation was first noticed in the late 1990s, it was not until 2004 that Oaks *et al.*. linked the vulture deaths to the veterinary use of diclofenac, a non-steroidal anti-inflammatory drug (NSAID) through drug exposure in their food source (Oaks *et al.*., 2004). This toxicity has since been reproduced under controlled experimental conditions in both captive Oriental and African white-backed vultures (Oaks *et al.*., 2004; Swan *et al.*., 2006b).

When exposed to diclofenac, the vultures all demonstrated similar clinical signs. The birds became depressed at approximately 24h after exposure, and became progressively more depressed until they succumbed 36 to 48h post-exposure (Swan *et al.*., 2006b). Similar signs have also been described in the field where sick birds were observed to be very depressed in their nests and "literally fall over dead" (Prakash *et al.*., 2003). The post mortem examinations were also very similar in both the tested and naturally poisoned birds with signs of a severe diffuse visceral gout and concurrent severe bilateral nephrosis being present (Meteyer *et al.*., 2006).

Although the cause of toxicity has been clearly linked to the veterinary use of diclofenac the mechanism of drug toxicity remains unclear (Meteyer *et al.*., 2005). At present numerous mechanisms of toxicity have been described for diclofenac in mammals and range from simple COX mediated ischemic necrosis to caspase induced liver failure. Although the toxicity seen may be as a result of inhibition of the COX enzyme, meloxicam, also a NSAID, showed no ill effects in the *G. africanus, G. bengalensis and G indicus* vultures when administered as either a formulated drug or as residues in meat from treated cattle (Swan *et al.*., 2006a). In people

meloxicam and diclofenac are both considered more cyclo-oxygenase (COX) II selective in their mechanism (Burke *et al.*., 2005). Pharmaceutically the only difference between the two molecules is their actual structure which tends to suggest that toxicity may be related to receptor binding affinity or perhaps even the plasma pharmacokinetics.

The aim of this study was to validate the fowl as a model for the study of diclofenac-induced intoxication in vultures to allow for future evaluations of the mechanism of toxicity. In addition to comparing clinical signs of toxicity, the pharmacokinetics, approximate lethal dose and changes in blood chemistry were compared between the two species.

5.3 Material and Methods

5.3.1 *Animals*

5.3.1.1 Husbandry

Leghorn layers were selected for this study because of easy availability. The layers were reared by a commercial rearer until 18 weeks of age after which they were allowed a week of acclimatisation, prior to commencing the study. They were housed in poultry houses of the Poultry Reference Laboratory, at the Faculty of Veterinary Science (Onderstepoort, Pretoria) and fed a commercial layer diet with water available *ad libitum*. Housing involved natural ventilation and an artificial light source of 18 hours. For the first two toxicity studies the fowls were housed in individual pens and wire cages, respectively. For the pharmacokinetic studies they were housed in layer cages in groups of three. For all studies the individual fowls were marked with an easily identifiable ring band with a unique colour code.

5.3.1.2 Intramuscular toxicity study

Thirty layers at the beginning of their production cycle were injected intramuscularly in the pectoral muscles. The layers were divided into groups (n=6) and were administered incremental doses of 0.6, 1.25, 2.5, 5 and 10 mg/kg. Dosing was staggered across 5 weeks

with one group treated per week. A non-treated control group was included for each dose level.

5.3.1.3 Pharmacokinetic study

Eighteen layers were used in a pharmacokinetic study and were initially dosed by the oral route, then the intramuscular route after a washout period of two weeks. Prior to the intramuscular study the animals were deemed clinically healthy and had normal serum uric acid concentrations. Diclofenac was administered at a dose of 0.8 mg/kg, the dose reported to kill African white-back vultures at probability of > 80% (Swan *et al.*., 2006b). According to our probit analysis of the toxicity data, the dose had a less than 10% possibility of inducing toxicity in fowls.

5.3.1.4 Blood collection

Blood was collected from the wing vein using a 21G needle and 2.5 ml syringe (Braun). Collected samples were immediately transferred into either plain or heparinised vacutainer tubes for serum chemistry analysis and diclofenac quantification, respectively. For the pilot and intramuscular toxicity study, samples for clinical pathology and diclofenac plasma concentrations were collected prior to dosing, 24 hours and at 48 hours post-dosing or death.

5.3.2 *Clinical Pathology*

The electrolytes Na^+, K^+ and blood pH were measured with a blood gas analyser (Rapidlab 34E Chiron diagnostics, Bayer SA). Uric acid and calcium (Ca^{2+}) were measured with a Nexet Chemistry Analyser (Alfa Klasserman, Bayer SA).

Serosal deposits of the white amorphous material were collected and tested for the presence of uric acid using the murexide reaction (Lumeij, 1994). For this reaction egg albumin (sulphur containing proteins) was used as a negative control (yellow) and urine urate deposits as a positive control (mauve).

The total accumulative increase in serum uric acid was calculated by plotting serum uric acid concentrations against time. The area under the curve was determined using the trapezoidal method (Toutain *et al.*., 2004).

5.3.3 *Pathology and Histopathology*

Lesions seen at necropsy were recorded. Tissue samples collected for residue analysis (liver, kidney, heart, brain, muscle) were frozen or preserved in buffered formalin for histopathology (liver, kidney, heart, spleen, shell gland, lungs). Samples collected in formalin were trimmed, embedded in paraffin, sectioned and stained with hematoxylin and eosin using standard methods.

5.3.4 *Residue Analysis*

Tissues harvested from the dead birds dosed at 10 mg/kg were analysed for the concentration of the diclofenac parent molecule in the tissues. Organs were stored at -30°C until analysed. Half a gram of defrosted tissue was homogenised, using a scalpel blade, and subsequently extracted with 2 ml acetonitrile, using a multitube vortex for ten minutes (Swan *et al.*., 2006a). The mixed samples were centrifuged at 4500 x G for 10 minutes, the supernatant was decanted, dried under a steady stream of nitrogen in a water bath at 50°C and stored at -25°C until analysis by high performance liquid chromatography (HPLC).

5.3.5 *Pharmacokinetic Analysis*

5.3.5.1 Model Parameters

For this study the third group of fowls were bled using a dose pooling method, where three birds were grouped to provide a single complete pharmacokinetic profile (Animal unit)(Ette *et al.*., 2004a; Ette *et al.*., 2004b). Each bird assigned to the animal unit was treated with drug at 0h and thereafter bled in rotation. A total of six profiles were used (18 birds). Blood samples were collected at 30, 45 minutes and at 1, 2, 3, 4, 5, 7, 9, 12 and 24 hrs for each profile. An

additional 5 minute bleed was included for the intramuscular profile. The same group of fowls were used for both profiles to allow intra and inter-group variations to be carried across both profiles. The birds were allowed a two week acclimatization period prior to conducting the oral study. A two week washout period was allowed prior to characterising the intramuscular profile. Compartmental pharmacokinetic analysis was undertaken using WinNonLin Ver 4.2 (sponsored by the PharSight Corporation.)

5.3.5.2 Sample preparation

Blood samples were centrifuged at 2800 x G for 15 minutes within 1 hour of sample collection. Sample volumes of 1.5 ml plasma were pipetted into 10ml tubes; 3ml diethyl ether was added to each tube, followed by 3ml of 0.3 M potassium dihydrogen phosphate, pH 3.5. The tubes were capped and the contents mixed for two minutes on a multi-tube vortex mixer, and centrifuged for 10 minutes at 2800 x G. The test tubes were inserted in an ice bath (methanol/solid carbon dioxide) to solidify the aqueous phase, and the organic layers decanted into 10 ml glass tubes. The organic layer was evaporated to dryness for 30 minutes at 50 ºC under a mild flow of nitrogen. The residue was dissolved in 400 µl mobile phase and transferred to the inserts, fitted in the vials, and the caps screwed on (Odensvi *et al.*., 1995).
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5.3.5.3 High performance liquid chromatography

A Beckman System Gold HPLC consisting of an autosampler module 507, programmable solvent module 126, diode array detector module (DAD) 168, and 32 Karat™ software package, was used (Beckman Instruments, Fullerton, California, USA). Separation was achieved with a Synergi Max-RP column (80A, 150 x 4.6 mm, 4; Phenomenex, Torrance, California, USA). The mobile phase consisted of 0.05M sodium dihydrogen phosphate (pH=4.85 to 4.89):CH_3CN, 42.5:57.5. 100 µl of the reconstituted samples were injected onto the HPLC column at 1 ml/min in an isocratic run. Detection of diclofenac and flunixin (internal standard) was carried out at 275 nm. The total runtime per sample was 8 minutes with retention times as follows: flunixin at 3.9 min, diclofenac at 4.9 min. Control values showed a mean % CV of 0.156 and regression coefficients greater than 0.99 for each

analytical run. The LLQ was established at 0.1µg/ml and a linear relationship between concentration and peak area was demonstrated for the total concentration range between 0.1 and 20µg/ml (Odensvi and Johansson, 1995).

5.3.6 *G. africanus results*

Stored serum samples collected during the diclofenac toxicity study were analysed for the concentration of potassium using the same method described above (refer to 5.3.2). Diclofenac plasma concentrations were re-analysed using the same pharmacokinetic model described above (Swan *et al.*., 2006b). To obtain a complete profile, the Cmax was set to 5 hours as this was the maximum concentration observed in each bird.

5.4 Results

5.4.1 *Clinical Signs*

Thirty birds in groups of six were exposed to five different doses of diclofenac. In total 9 birds died from severe toxicity while the tenth bird, at the highest dose, showed signs of toxicity but eventually recovered. None of the control birds died. Two birds died in each of the 1.25, 2.5 and 5 mg/kg group with the three remaining birds dying in the 10 mg/kg group. The sick birds (n=10) were reluctant to move, perched in one corner of their cages, appeared to be asleep and had stopped eating (i.e. were severely depressed). The birds initially responded to external stimuli such as touch and noise by “waking up” but within a minute became depressed once again. This depression on average occurred at 24h post-dosing and progressively became more severe. Prior to death, the affected birds appeared to be comatose and could not be roused. In the birds that were producing eggs, at 24 hours the eggs were malformed and very thin shelled. Although the shell weight had decreased, the ratio of shell weight:thickness remained constant.

5.4.2 *Clinical Pathology*

Uric acid, Ca^{2+}, Na^{+} and K^{+} were within the normal range at 24h for that published for the fowl (Ross *et al.*., 1978). After 24h, only plasma uric acid concentrations were extremely high (7.662 ± 2.54 µg/ml) for the 9 birds that eventually died. For the period from dosing to mortality the dead birds exhibited concentrations of uric acid of 234 ± 34 h*µg/ml. The sick bird that recovered was exhibited 157 h*µg/ml uric acid. The blood pH at 24 h was 7.44 but decreased to ± 6.7 at death. The drop in pH had a corresponding increase of plasma potassium exceeding 15mmol/l (25.3 ± 10 mmol/l).

5.4.3 *Necropsy*

Severe visceral gout and severe diffuse nephrosis was evident in only the birds that died. The birds in the 5 mg/kg and 10 mg/kg dose group also had signs of articular deposition of a white amorphous substance. In the birds necropsied within minutes of dying, the diffuse white substance within the pericardium was in a liquid form and solidified as the carcass cooled to room temperature. Samples of the white material on the viscera were analysed for the presence of uric acid. For the majority of samples collected, a reaction was achieved for the presence of sulphur containing protein and not uric acid. Only one sample analysed from the highest dose group eventually tested positive for uric acid. The specific sample was from the pericardial effusion. When this sample was allowed to cool, it finally crystallised and supported the histopathological diagnosis of urate tophi. No signs of pathology were evident in the healthy birds euthanized five days after dosing.

5.4.4 *Histopathology*

The cortical architecture of the kidney showed marked disruption due to the presence of tophi containing both spiculate as well as globloid urate forms which caused both cell necrosis and tubule loss. In most cases massive heterophil infiltration into the interstitium of the cortex as well into the tubule and collecting ducts was present.

Renal necrosis was, however, most severe in the birds that had died in the 10 mg/kg group and was characterized by massive urate crystal precipitation with necrosis of the adjacent structures. In most cases, although the outline of the tubules was still apparent, most of the tubular structure was lost due to the presence of large aggregates of urates (Figure 5-1). In two of the birds the tophi had multinucleate giant cells at their periphery. Some areas of the interstitium were associated with macrophage and lymphocyte infiltrate and possible early fibroblast proliferation.

The liver was also severely affected, and was characterized by small tophi associated with hepatocyte necrosis. In most cases the tophi were associated with an inflammatory reaction of both heterophils and round cells. In some cases the tophi consisted of central aggregates of urate spicules with minimal infiltration of inflammatory cells at the periphery.

The shell gland had scattered tophi present within the gland resulting in local necrosis of the glands as well as the overlying epithelium. The heart, lungs and spleen contained scattered tophi within the parenchyma which was associated with mild to more extensive cell necrosis. At times aggregates consisted of only urate deposits which had not yet become tophi.

The diclofenac plasma concentrations measured in the dosed fowls were very variable. Only seven fowls, from the 30 treated, had detectable drug concentrations at 24 h post dosing, with a mean of 0.20 ± 0.05µg/ml. Only one fowl (5 mg/kg group) had detectable drug concentration at 48 h which translate to a half-life of 14 h. None of the other animals, sick or healthy, had any detectable drug at the last sampling point indicating a half-life under 5h (± 5 half-lives).

There was no relationship between animals demonstrating plasma concentrations at 24h and the occurrence of toxicity. Only two of the dead animals had detectable tissue drug concentrations in either the kidney or liver. The specific tissue partition co-efficient could not be determined due to the absence of a relationship with diclofenac plasma tissue concentrations.

5.4.5 *Pharmacokinetic (PK) Analysis*

All six animal units were best analysed using a one compartmental open model. The PK parameters obtained is listed in the Table 5-1 for both routes and the average plasma profile in Figure 5-2. The drug showed a relatively short half-life of elimination of 0.6 ± 0.19 h following intramuscular administration and 0.89 ± 0.36 h following oral administration. None of the animals in this phase of the study showed any signs of toxicity.

5.5 Discussion

5.5.1 *Comparison Between Fowls and G. Africanus Vultures*

The clinical signs and increase in uric acid concentrations evident in the fowls succumbing to toxicity was similar to the signs reported by Swan *et al.*. (2006b) for *G. africanus*. Similarity in total uric acid exposure between fowls that had died and those of the vultures (calculated from the results presented by Swan *et al.*. 2006b) were observed. The total uric acid exposure for vultures was calculated as 219 ± 1 mmol/L/h and suggests that the extent of uric acid exposure is directly related to mortality (Figure 5-3).

A terminal increase in potassium concentrations evident in the dead fowl's plasma prompted re-analysis of stored samples from the *G. africanus* vultures for comparison (Swan *et al.*., 2006b). A corresponding increase in the plasma potassium concentrations was present in vultures. In the past it has been suggested that a terminal increase in potassium was the cause of death in gouty fowls (Lumeij, 1994). This study therefore supports this supposition that the

cause of death was potassium related. Hyperkalaemia is a known cause of bradycardia in birds (Zandvliet, 2005).

On comparison the pathological changes observed in the Leghorn layers following diclofenac exposure were similar to those reported in the Gyps species, i.e. diffuse visceral gout to severe nephrosis (Oaks *et al.*. 2004, Meteyer *et al.*. 2005, Swan *et al.*. 2006b). Differences were, however, present on histopathological examinations. The fowl kidneys and other organs in the layers appeared to be more severely affected than in the vulture. In addition the tophi in the layers were associated with a fairly marked cell infiltration. In vultures, minimal (or more usually) no cellular response was evident. This tends to suggest that the period of time from tophi formation to death was more prolonged in the layers providing more opportunity to launch a cellular response. However, the average time to death between vultures and layers were very similar (42 hours in vultures and 44.5 ± 6.6 hours in fowls).

This toxicity study confirmed the greater tolerance of fowls to diclofenac poisoning compared to Gyps vultures. A probit analysis (SPSS 13, SPSS Inc.) of the toxicity data showed a LD_{50} of 9.8 mg/kg for the intramuscular dose, which is markedly higher that the oral LD_{50} calculated for *G. bengalensis* of 0.098 to 0.225 mg/kg (95% CI 0.027 – 0.351 mg/kg)(Swan *et al.*., 2006b). Although the routes of exposure were different, the pharmacokinetic data generated allows for a better comparison. The bioavailability of diclofenac in fowls following oral administration was 50% compared to the intramuscular route with a relative bioavailability of 50%. This indicates that the oral LD_{50} in the layers could be as high as 19.6 mg/kg on the assumption that the dose-response relationship is linear. The intramuscular pharmacokinetic data does, however, have to be interpreted with care, as the same fowls were used for both phases of the study. As such it is possible that mild, clinically overt, cellular toxicological changes induced by the oral dosing may have been carried through to the intramuscular phase.

Although only an incomplete diclofenac plasma concentration versus time profile is available for diclofenac in vultures, the two *G. africanus* vultures dosed at 0.8 mg/kg still had a five fold higher plasma concentration at 5 hours than fowls. The natural logarithmic (Ln) transformed data (Figure 5-4) generated by Swan *et al.* (2006b), suggests that the time to maximum

concentration (Tmax) for vultures is approximately 5 hours as the graph is no longer linear at this point. By assuming that 5 hours is the Tmax we estimate an AUC of 34 and 133ugml/h for vultures 1 and 2, respectively (Table 5-2). When comparing the overall extent of absorption (AUC), at 0.8 mg/kg, the *G. africanus* vultures had a greater overall exposure than any of the fowls treated, even though the vultures were treated orally and the fowls intramuscularly (Swan *et al.*, 2006b). With the AUC being a measure of the extent of absorption, it clearly evident that diclofenac is absorbed to a greater degree in the vulture than the fowl.

The other major pharmacokinetic difference observed was in the biological half-life of diclofenac between fowls and vultures. Biological half-lives of 14 and 18 hours were reported for the two vultures (Table 5-2)(data from Swan *et al.*, 2006b). This was markedly different to fowls which had a half-life of approximately 1h. These differences were also reflected in the tissue concentrations of diclofenac, which was present in all the major organs (liver, kidney, heart, brain and muscle) of the orally dosed *G. africanus* vultures and almost completely below the level of quantification in the intramuscularly dosed fowls, even at the maximum dose. The volume of distribution was much smaller in vultures than for fowls, while the fowls had a volume of distribution more typical of that described in mammals (Busch *et al.*., 1998). Clearance was similarly lower in vultures than for fowls. This possibly suggests a larger degree of plasma protein binding in the vulture with a higher concentration of drug remaining in the plasma.

When comparing intestinal absorption, the absorption half-life appeared to be much longer in the vultures than in fowls, and is similar to the elimination half-life found in fowls. It may therefore be plausible that flip-flop kinetics occurs in vultures with diclofenac being slowly released from the crop over time. This hypothesis is supported by results from another pharmacokinetic study in fowls that showed the absorption of ibuprofen was delayed when the drug was deposited into the crop instead of the proventriculus (Vermeulen *et al.*., 2001).

5.5.2 *Comparison of Results to That Presented by Reddy et al. (2006)*

The 33% mortality seen at the 5mg/kg dose was similar to 40% described by Reddy *et al.*.(2006) However, the necropsy and histological findings are very different to that discussed in their report. In contrast we found that toxicological changes in the fowls were in-fact similar to those for the vultures as discussed above. Reddy *et al.*, also discussed the changes as being renal necrosis accompanied by tubular fibrosis. The presence of fibrosis indicates a more long-standing condition which is probably due to the long survival time experienced. The lack of renal fibrosis in our study is more in line with the findings by Oaks *et al.*. (2004) in which no signs of fibrosis or chronic changes were found. Further studies will be needed to investigate why the pathology of the acute deaths differed to such an extent between the trials.

5.5.3 *Suggestive Pathophysiology of the Clinical Signs*

The clinical signs seen in all the birds may be explained by the changes in the clinical pathology. The metabolic acidosis (pH of 6.5) can be linked to an increase in plasma uric acid levels and may be further exacerbated by the inability of the damaged proximal convoluted tubule to conserve bicarbonate (Seifter, 2004). At 24 hours this is countered by the natural buffering systems as seen with the normal blood pH (Campbell, 2004). The pH of 6.5 at the point of death indicates a progressive drop in the blood pH from 24 hours to the time of death.

Acidosis also explains the progressive increase in CNS depression in that depression corresponded to the initial increase in uric acid and progressively became more severe until death. The clinical signs of acidosis in cattle provides further credence to this theory as one of the major signs seen in these animals is initial depression which progressively becomes more severe until the animal dies (Blood *et al.*., 1989). Similarly severe plasma acidosis is characterised by depression in people (Seifter, 2004).

The actual cause of death in the birds is once again most likely attributable to the terminal hyperkalaemia. The massive potassium increase would lead to cardiac failure and death as

potassium is very finely controlled in all animals. An increase in potassium will lead to an abnormal cardiac rhythm by altering the T-wave and at high levels will lead to fatal cardiac arrhythmias (Sturkie, 1986; Zandvliet, 2005).

5.6 Conclusion

The clinical signs of severe depression and death in the fowls and *G. africanus* vultures appeared to be directly related to the increase in uric acid in the plasma and possibly to hyperkalaemia. With the cause of death being clearer, it may be possible to treat affected birds. From the one fowl that recovered, we know that diclofenac toxicity is not uniformly lethal once clinical signs develop. In addition to gavaging large quantities of water to flush out the effected kidneys, other treatments may be equally as effective. At this stage we would suggest an immediate correction of the acid base status of the animal. If stabilised it should prevent the terminal increase in potassium while treatment with a hydrochlorthiazide diuretic may also enhance the excretion of potassium in the urine thereby preventing the lethal hyperkalaemia (Sturkie, 1986) assuming that the potassium exchange in the distal tubules is still functional.

With dose being the only major difference in the toxicity of the compound, it is proposed that fowls could be used as a model for further studies into the pathophysiology of diclofenac induced intoxication in vultures. The difference in dose does, however, imply that fowls are not suitable as a model to determine the susceptibility of the vulture to other veterinary NSAIDs. With the present study evaluating only the acute toxic effect of diclofenac in fowls it will be of benefit to study the toxic effects of diclofenac at reduced doses following chronic exposure.

5.7 Acknowledgements

This research project was approved by the animal use and care committee of the University of Pretoria. This project was made possible by the generous support of the Royal Society for the Protection of Birds and their public donors.

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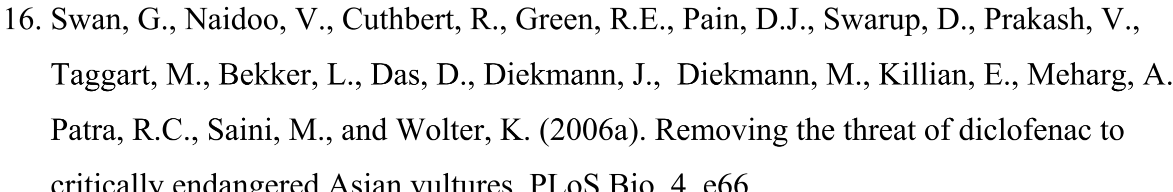

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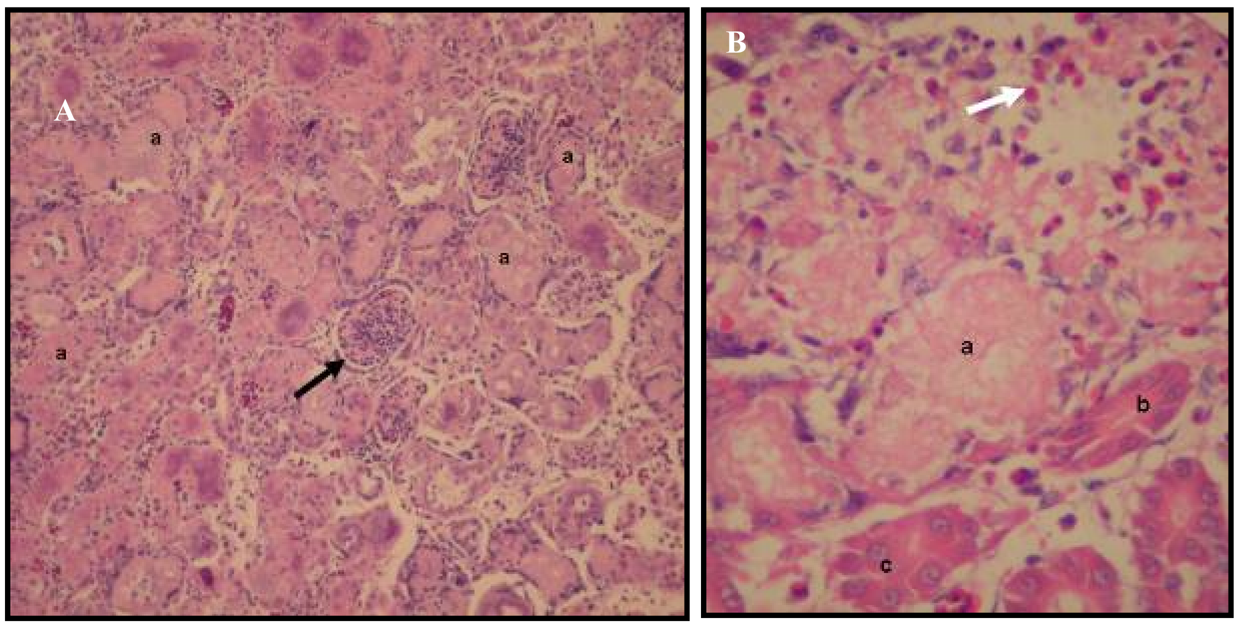

Figure 5-1: Kidney. A: HE x100 B: HE x 300 from a fowl dosed at 10 mg/kg. Marked tubular damage with complete destruction of the tubular structure (a), tubule with minimal damage (b) and normal tubule (c). Heterophil infiltration varied (white arrow). Unaffected mammalian glomerulus (black arrow).

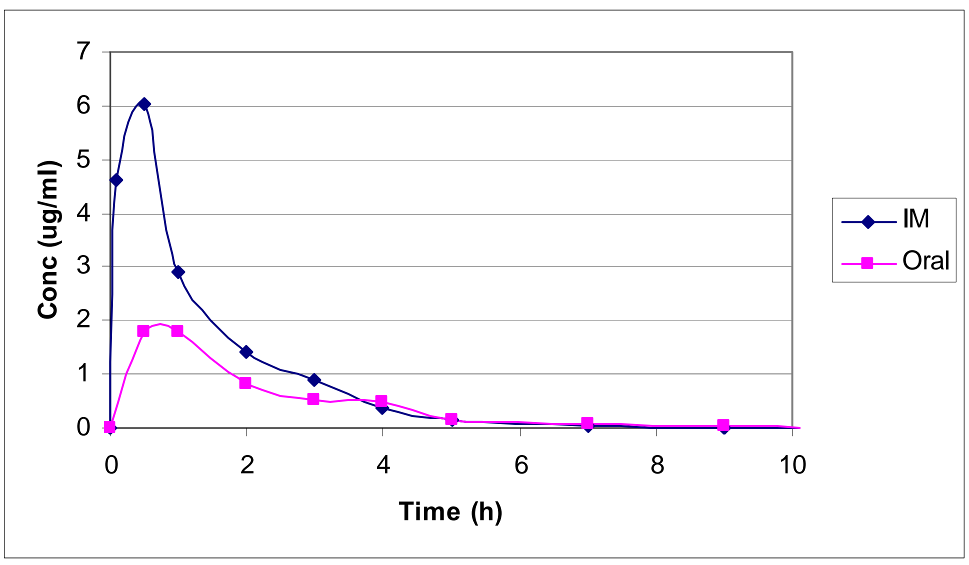

Figure 5-2: Mean diclofenac plasma concentration versus time profile for the oral and intramuscular route of administration

Table 5-1: Pharmacokinetic parameters following oral and intramuscular administration of diclofenac at 0.8mg/kg to fowls

Units		Intramuscular			Per Os		
		Mean	SD	%CV	Mean	SD	%CV
AUC	h*µg/ml	8.51	1.98	23.22	4.33	0.74	17.07
Cl/F	ml/h/kg	98.42	23.21	23.58	189.45	33.16	17.5
Cmax	µg/ml	6.79	0.87	12.78	2.11	0.63	29.98
A	1/hr	9.71	3.12	32.12	4.37	3.93	89.84
Alpha-HL	h	0.08	0.02	27	0.31	0.23	74.31
B	1/h	1.15	0.42	36.91	0.87	0.28	32.57
Beta-HL	h	0.66	0.20	30.02	0.89	0.37	41.37
Tmax	h	0.26	0.04	16.33	0.63	0.28	44.24
Vd/F	L/kg	0.09	16.00	17.89	0.24	85.23	35.73

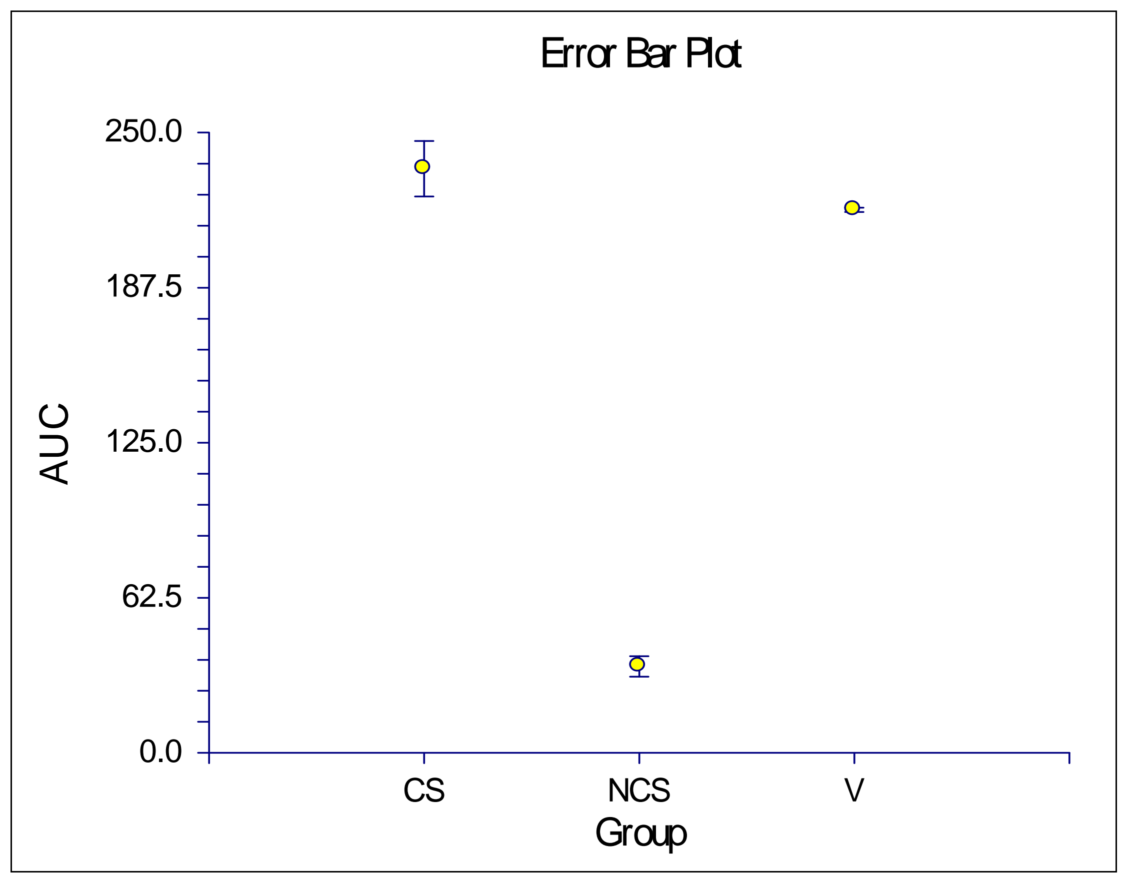

Figure 5-3: Mean uric acid levels for the bird that died (CS), treated birds that survived (NCS) and the two *G. africanus* vultures (V)

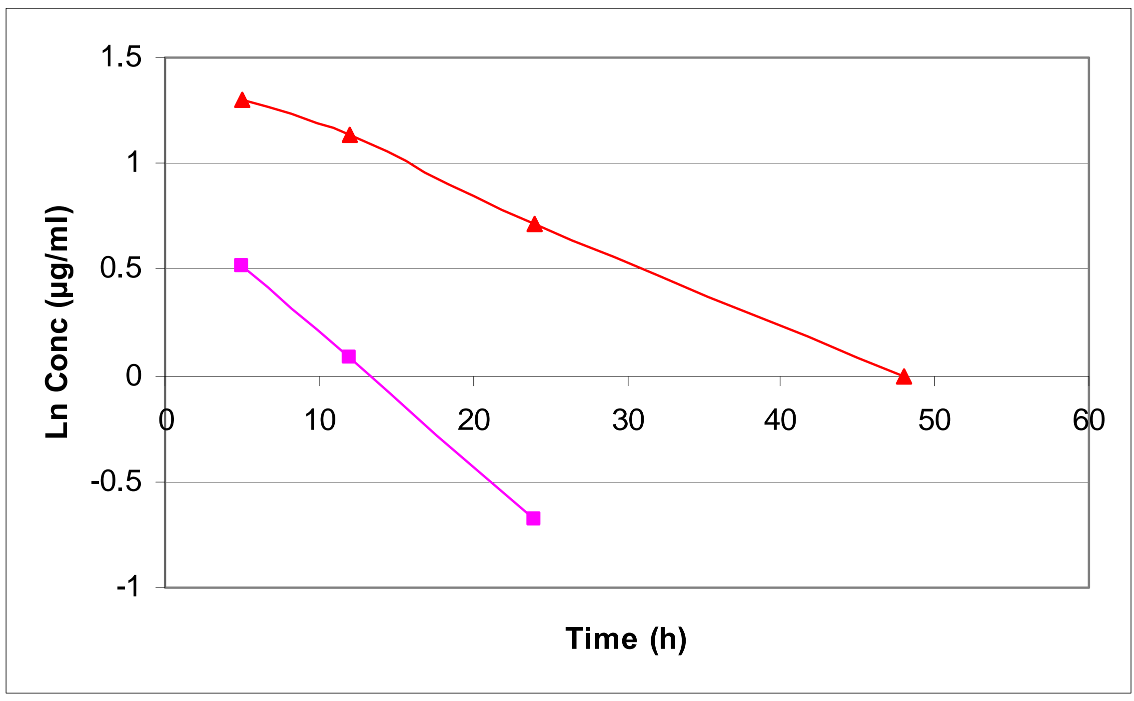

Figure 5-4: Semi-logarithmic plasma profiles for diclofenac from the two treated vultures (courtesy of Swan *et al.*, 2006)

Table 5-2: Estimated PK parameters of two dosed vultures. Values were calculated on the assumption of Cmax being achieved at 5h

Parameter	Units	Vulture 1	Vulture 2
AUC	ug/ml/h	34.09	133.44
Cl/F	ml/h	0.02	0.01
Cmax	ug/ml	1.76	3.68
K01	h^{-1}	0.85	0.63
K01-HL	h	0.81	1.11
K10	h^{-1}	0.06	0.03
K10-HL	h	10.90	21.39
Tmax	h	3.29	4.99
Vd/F	L/kg	0.0004	0.0002

CHAPTER 6: Establishment of selected baseline blood chemistry and hematological parameters in captive and wild-caught African White-backed vultures (*Gyps africanus*)

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ESTABLISHMENT OF SELECTED BASELINE BLOOD CHEMISTRY AND HEMATOLOGICAL PARAMETERS IN CAPTIVE AND WILD-CAUGHT AFRICAN WHITE-BACKED VULTURES (*GYPS AFRICANUS*)

Naidoo V[1]†, Diekmann M[2], Wolters K[3], Swan GE[1]

[1] Faculty of Veterinary Science, University of Pretoria, South Africa

[2] REST, Namibia

[3] De Wildt Cheetah and Wildlife Trust, South Africa

6.1 Abstract

Following the devastating collapse of three vulture populations on the Asian Sub-continent as a result of their exposure to diclofenac, it has become necessary to establish the mechanism of toxicity of this veterinary medicine to vultures. In doing so, it is hoped that other potential hazards may be identified and removed from their environment. At this stage little information is available on the normal physiology of the white-back vulture, making it difficult to interpret the changes that occurs following diclofenac toxicity. The aim of this article was to establish baseline parameters for hematological and selected serum chemistry parameters for *Gyps africanus*, the model validated for further studies into diclofenac toxicity. Captive non-releasable and wild captured vultures were used to determine the reference values. The hematology values measured were erythrocyte counts, hemoglobin concentration, hematocrit, packed cell volume, mean corpuscular volume, mean corpuscular hemoglobin concentration and total and differential leukocyte counts. The chemistry analytes measured included sodium, potassium, calcium, albumin and globulin concentrations, and aspartate aminotransferase (AST), creatine kinase (CK) and alanine aminotransferase (ALT) activities. Uric acid and urea concentrations and the urea:uric acid ratio were also evaluated. Values are presented as means, standard deviations and reference intervals. The serum chemistry parameters selected may provide a

† Private Bag X04, Section of Pharmacology and Toxicology, Onderstepoort, 0110 South Africa. vinny.naidoo@up.ac.za

starting point for the evaluation of changes in renal and hepatic function, the organ systems most severely affected by diclofenac, following toxicity. The results are also compared to that for *G. africanus* nestlings. In comparison with the nestlings it is evident that the clinical pathological parameters are age related. This indicates that the use of nestling values for the evaluation of adults' clinical pathology may be unreliable and could lead to incorrect assumptions.

Key Words

Clinical pathology, vultures, African White Back vultures, Hematology, serum chemistry, *Gyps africanus*

6.2 Introduction

In the recent past the dramatic decline in vulture numbers across the Indian subcontinent has illustrated the impact a simple chemical compound could have in the environment (Prakash *et al.*., 2003, Green *et al.*., 2004, Oaks *et al.*., 2004). Populations of Oriental white-backed vultures (*Gyps bengalensis*), long-billed vultures (*G. indicus*) and slender-billed vultures (*G. tenuirostris*) in the region have markedly decreased by more than 98% following exposure to the non-steroidal anti-inflammatory drug (NSAID) diclofenac over an estimated fifteen year period (Prakash *et al.*., 2003, Green *et al.*., 2004, Oaks *et al.*., 2004). At present the Oriental white-backed vulture has been the most affected of the species. This drug exposure resulted purely from drug residues within livestock carcasses left out to feed the vultures. The drug has an estimated LD_{50} in the range of 0.098 to 0.225 mg/ kg (Swan *et al.*., 2006), which following exposure results in death within a few days. All the dead birds showed signs of renal failure, liver injury and diffuse visceral gout on post-mortem examination (Oaks *et al.*., 2004, Swan *et al.*., 2006). In 2006 Swan and co-workers showed the African white-backed vulture (*G. africanus*) to be as susceptible to diclofenac as its oriental cousins (Swan *et al.*., 2006) and therefore an adequate model to evaluate the toxicity of diclofenac.

Although detailed information is available for blood biochemistry, hematological and histopathological changes following NSAID toxicity, relatively little information is available to describe normal values for white-backed vultures (Oaks *et al.*., 2004, Shultz *et al.*., 2004, Swan *et al.*., 2006). This knowledge is vital to ascertain the reason for the

species' susceptible to toxicity as well as to identify other potential toxic NSAIDs. At present the only available published article describes hematology and blood biochemistry values for African White-backed vultures (AWBV) nestlings in South Africa (van Wyk *et al.*., 1998). Although immensely useful, normal values for nestlings are not necessarily representative for adult birds in which diclofenac toxicity is most likely to occur.

In this study we report baseline hematological and blood chemistry values for adult African White-Backed Vultures. The parameters selected will allow for a further evaluation of pathophysiology of diclofenac toxicity as they are indicators of hepatocellular injury and renal function, the two organs most severely affected by diclofenac toxicity.

6.3 Materials and Method

6.3.1 *Collection of blood samples*

Wild birds (n=25) were caught in Otjiwarango, Namibia, by the Rare and Endangered Species Trust (REST) during their routine ringing project in January 2004 and April 2005 respectively. Blood was collected by venipuncture from either the brachial or tarsal vein into sterile syringes with 25 gauge needles and subsequently transferred into evacuated EDTA and serum tubes (Campbell, 1984). Serum samples were spun down at 2000 rpm, and stored at -30 ºC until analysed. With the exception of one subadult, all birds captured were adults. The sex of the birds was unknown due to the difficulty in sexing them.

To establish the full hematological profile captive non-releasable birds (n= 21) were also sampled. The birds were obtained from the De Wildt Cheetah and Wildlife Trust and from the Pretoria Zoological Gardens (PZG). The birds from De Wildt were mainly rescued following pylon injuries and were non-releasable due to amputations resulting from their injuries. The PZG birds were healthy captive breeding stock. In the captive population, 10 were adults while the rest were sub-adults. The sex of the birds was unknown. To minimize the potential influence of latent disease the birds included in the analysis were deemed healthy based on normal habitus, normal appetite, being of average weight and condition for the species, and a history of being in captivity under daily supervision for at least two months prior to induction into the study. One week prior to the sample collection

the captive birds were transferred from a communal aviary into individual cages of 2x2x2m to minimize the effect of catching and the related corticosterone release on the hematological profiles. Blood was collected in a similar manner to that used in wild-caught birds. All non-releasable birds were sampled on three different occasions in August 2004, November 2004 and January 2005.

In addition to the collection of a single sample for baseline determination, two additional non-releasable birds were subjected to a series of blood collections before and 4, 8, 12 and 24 hours after the feeding of 200g of fresh meat to allow for interpretation of the U:UA ratio described for the wild-birds (Lumeij, 1994).

6.3.2 *Hematology*

Thin blood smears were prepared from the EDTA samples by means of the slide on slide technique (Campbell, 1984). Smears were subsequently air-dried and immediately stained with Diff-Quik (Kyron laboratories, SA). The smears were evaluated for cell morphology and the presence of parasites and a differential leukocyte count was performed. The total leukocyte count (WBC) was evaluated using an improved Neubauer haemocytometer with Turck's fluid (Kerr, 2002)

All other haematological parameters were analysed by an automated cell counter (CellDyn 3700, Abbott). These were: total erythrocyte counts (RBC), hemoglobin concentration (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and thrombocyte counts. The hematocrit was confirmed using the micro-hematocrit method [Packed cell volume (PCV)] described in the literature (Campbell). The MCV was derived automatically by the cell counter. The Hct was calculated from the following equation: Hct=RBC x MCV / 10. The MCHC was calculated as: Hb/Hct x 100.

6.3.3 *Serum Chemistry*

The electrolytes calcium (Ca^{2+}), sodium (Na^{+}) and potassium (K^{+}) were measured with a blood gas analyser (Rapidlab 34E Chiron diagnostics, Bayer SA) using an ion selective

electrode (ISE) method. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine kinase (CK) activities and uric acid (UA), urea, total protein and albumin concentrations were measured with a Nexet Chemistry Analyser (Alfa Klasserman, Bayer SA). Globulin values were calculated as the difference of the total protein and albumin concentrations. The urea:uric acid (U:UA) ratio was calculated by the following equation: urea (mmol/l) x 1000:uric acid (µmol/l) (Lumeij, 1994).

6.3.4 *Statistical Analysis*

Statistical analysis was performed with the statistical software SPSS13 (SPSS Inc.). A Kolmogorov-Smirnov and Lilliefors table was used to assess the normality of distribution (Marco *et al.*., 2000). Reference intervals were calculated as arithmetic mean ± 2 SD for non-transformed data. Where normality was demonstrated using natural log transformed (Ln) data, the results are presented as geometric mean and SD. Confidence intervals for the Ln transformed data were back transformed as mean (Ln (X)) ± 2SD(Ln(X)), where X represents the clinical pathological parameter being evaluated (Bland et. al.,1996). Where normality could not be demonstrated, data was discussed using descriptive statistics. For comparisons significance was determined using the T-Test.

6.4 Results

The EDTA samples collected from the wild birds were not analyzable for full hematology due to a long delay in transporting samples from the remote area of collection. For these we are therefore only able to present the Hct which was done at the capture site. To compensate for this, the hematology was repeated in non-releasable birds. CK, Alb and ALT were also evaluated in the non-releasable birds to demonstrate normality.

The data for normally-distributed serum chemistry from the wild birds and hematology values from the non-releasable birds are presented in Table 6-1 and Table 6-2. We are confident that these reference intervals will be relevant for the current population of White-backed vultures. Parameters for which normality could not be demonstrated are listed in Table 6-3.

6.5 Discussion

For this discussion, the results for the adult vultures are compared to the values published for nestlings of the same species (van Wyk *et al.*., 1998). The results by van Wyk *et al.*, only discuss results by range, mean and standard deviation and not as reference intervals.

6.5.1 *Erythron*

The PCV of the wild birds was 42 ± 4.06 and did not differ significantly to that of the non-releasable birds. The PCV and MCHC were similar between the adult birds and that reported for the nestlings. Both Hb and the RBC were higher in the adult birds than the 140 g/L and 1.4 x 10^{12} cells/L reported, respectively for the Gyps nestlings. Stress as the causative factor for the increased RBC can be ruled out as the avian spleen lack both storage capacity and a muscular capsule. Thus it makes it physiologically impossible for the avian spleen to inject red cells into circulation under stressful conditions such as blood sampling as seen in mammals (John, 1994, Latimer *et al.*., 2003).

It is, however, plausible that the level of activity of the birds may contribute to the differences. With vultures being some of the highest soaring birds it is possible that the lower oxygen levels of high altitudes combined with the activity of flight may have contributed to the increased RBC and Hb as a compensatory mechanism in the adult bird (Campbell, 1984, Satheesan *et al.*., 2000). This is supported by Carpenter (1975) who showed that strong fliers in general tended to have a naturally higher RBC. The mean MCV was higher in the adult Gyps than for the 161.30 fL reported in nestlings.

The Hct was also significantly higher than the PCV in all the birds. Although the exact cause of the difference is unknown, it is likely due to the anticoagulant used. Hematology samples were collected in K_3EDTA, which is known to induce an artifactual decrease in the PCV, while not affecting the Hct (Cott *et al.*., 2003). According to Abbot this decrease may be as high as 2-4% (Abbot, 2006).

6.5.2 *Leukon*

The mean WBC count was markedly lower than the 28.59 10^9 cells/L reported in the Gyps nestlings. Unlike other birds the segmented heterophil (H) was the predominant leukocyte in circulation (Joseph, 1999, Fudge, 2000, Aengwanich *et al.*., 2002). At this stage the only raptor species we could identify with the heterophil as the predominant leukocyte was the 61 % reported in Peregrine falcon nestlings (Del Pilar *et al.*., 2001). The leukogram was also characterised by the absence of immature (band) heterophils (Fudge, 2000). This is considered normal as their presence in the circulation is usually indicative of an inflammatory reaction or an infectious condition. The high mature heterophil count in the absence of immature heterophils may be an indicator of a stress or physiological leukogram. This is supported by Campbell (2004) who suggested that a WBC higher than 15 x 10^9 cells/L is indicative of stress in tame birds. As such it is possible that the reported high values for the WBC and heterpohils are false. No specific data is reported for the Gyps nestlings.

6.5.3 *Plasma proteins*

Albumin concentrations were similar to the Gyps nestlings. The similarity would indicate that serum albumin concentration is finely controlled from an early age and is most likely related to the close relationship between albumin and plasma oncotic pressure (Coles, 2005). The mean globulin concentrations were higher in the adults than the 16.6 g/L reported in the nestlings and is most likely related to increased immune competence resulting from a difference in environmental antigenic stimulation.

6.5.4 *Plasma Electrolytes*

The values for sodium and potassium did not show normal distribution and are presented as 95% confidence intervals. Sodium concentrations were higher than the 130 mmol/L reported in Gyps nestlings. The cause of this increase is unknown. Although dehydration

may be a possible cause, the value may be a true reflection for adults as the concentrations were within the range of 140 to 160 mmol/L reported for three other raptor species (Joseph,1999, Lierz, 2003).

The mean potassium value was nearly 10 % higher than the 1.29 mmol/L reported in Gyps nestlings but was still within the range of 0.6 to 3.2 mmol/L reported in other raptors (Joseph, 1999).

6.5.5 *Enzymes*

With CK being associated with muscle trauma, we minimized injury in the birds by keeping them in individual cages and as such these results are most likely a fair reflection of the normal values for the species. The results also fell within the range of 220 to 500 U/L reported for four other species of raptors (Joseph, 1999). The effect of muscle trauma on CK levels is evident in the wild birds which struggled endlessly in their attempts of escape from the aviary (Table 6-3).

AST was measured in only the wild birds and was much higher than the mean of 78 U/L reported in Gyps nestlings and is most likely linked to muscle injury. ALT and AST are not specific indicators of liver trauma as both these enzymes are distributed in other tissues, especially muscle (Joseph, 1999). As such, we would suggest that increased ALT and AST, in the absence of massively increased CK, would be indicative of hepatocellular injury.

6.5.6 *Urea and Uric acid*

Unlike mammals, urea is not a preferred pathway for the excretion of nitrogenous wastes in avians, even though it is fairly well regulated (Lumeij, 1994, Lierz, 2003). Nitrogenous excretion occurs mainly via uric acid (80 to 90%) from the proximal convoluted tubules. Although a change in either parameter may be an indicator of renal damage or dehydration, this change in function is best evaluated by the ratio of U:UA, where an increase is indicative of pre-renal azotaemia (reduced renal arterial pressure, perfusion or dehydration) while a decrease is indicative of renal damage.

A mean U:UA of 5.45 was present in the adult birds. This figure is unfortunately static and fails to consider changes in plasma uric acid brought about by feeding. To allow for an interpretation, the change in the ratio was evaluated in the two additional non-releasable vultures. Following a 24 hour fast this was 5.95 while after feeding the ratio was 4.74, 1.85 and 2.89 at 5, 12 and 24 hours respectively. It therefore appears that the value obtained in the wild adult birds represents the unfed state.

6.6 Conclusion

The results presented above are an important first step in describing the pathophysiology of diclofenac toxicity in the Asian White-Backed Vulture. Although the results for the Gyps nestlings were available as a reference, many of the parameters presented were not representative for adult birds.

6.7 Acknowledgements

Ethics approval was attained from the University of Pretoria's Animal Use and Care committee. This research project was made possible due to generous funding by the Royal Society for the Protection of Birds. Many thanks to the vulture capture team, Otjiwarango Namibia, for their invaluable assistance in capturing and handling the wild birds.

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Table 6-1: Reference hematology values for the captive White backed vultures (n=21)

Parameter	Mean	SD	Min	Max	Reference Interval		
Hb (g/L)	196.60	16.83	142.00	241.00	162.94	-	230.25
RBC (x 10^{12} /L)	2.55	0.22	2.01	3.03	2.11	-	2.98
Hct (L/L)	0.50	0.04	0.38	0.60	0.42	-	0.58
MCV (fL)	196.70	5.55	182.00	211.00	185.60	-	207.81
MCHC (g/dL)	39.3	1.53	35.40	42.00	36.25	-	42.35
WBC (x 10^9/L)	16.71	1..63	4.00	34.00	13.45	-	19.97
Hmat (x 10^9/L)	13.70	6.11	3.04	27.60	1.48	-	25.93
Himmat (Band) (x 10^9/L)	0.00	0.00	0.00	0.00	0.00	-	0.00
Lymph (x 10^9/L)	1.02	1.91	0.00	12.55	0.00	-	4.84
Mono (x 10^9/L)	1.57	1.04	0.10	5.78	0.00	-	3.65
Eos (x 10^9/L)	0.75	0.71	0.00	3.68	0.00	-	2.16
Bas (x 10^9/L)	0.00	0.00	0.00	0.00	0.00	-	0.00
PCV (%)	44.3	4.79	31.00	53.00	34.72	-	53.88

Table 6-2: Reference values for selected blood chemistry parameters in Wild (n=14) and Captive (n=25) African White-backed vultures

Parameter	Mean	SD	Min	Max	Reference Interval		
UA (mmol/L)‡	0.65	0.03	0.22	1.195	.0.58	-	0.724
Urea (mmol/L)‡	3.21	1.01	1.1	4.7	1.18	-	5.23
U:UA‡	5.47	1.39	2.2	12.7	1.39	-	2.69
Alb (g/L)*†	11.69	1.06	10.50	13.60	10.37	-	13.18
Globulin (g/L)†	21.78	1.14	16	29	16.76	-	28.30
ALT (U/L)*†	35.51	1.99	6.00	95.00	9.233	-	144.45
AST (U/L)†	1620.92	2.24	241	5120	238	-	6160
CK (U/L)*†	287.47	137.00	136.00	631.00	112.85	-	596.15

*Results obtained from the captive birds; †Results represent geometric mean and SD; ‡Samples represent arithmetic mean and SD

Table 6-3: Parameters from wild birds for which normality could not be established

Parameter	Mean	SD	min	max	LCI	UCI
Calcium (mmol/L)	1.71	0.77	0.77	2.87	1.49	1.93
K (mmol/L)	1.41	0.61	0.66	3.12	1.16	1.67
Na (mmol/L)	153.7	12.17	108	176	148.59	158.87
CK (U/L)	16910	14988	415	56080	10856	22964
Alb (g/L)	12.79	0.88	11	15	12.41	13.1
TP (g/L)	34.96	3.75	27	43	33.3	36.54

LCI: 95% Lower confidence interval; UCI: 95% Upper confidence interval

CHAPTER 7: Diclofenac in Gyps Vultures: A molecular mechanism of toxicity

The following manuscript has been submitted for consideration in Nature: Cell biology

Diclofenac in Gyps Vultures: A molecular mechanism of toxicity

V Naidoo[5] & GE Swan

Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, South Africa

Diclofenac (DF), a non-steroidal anti-inflammatory drug (NSAID) was shown to have caused a massive decline in the vulture populations on the Asian subcontinent[1-4]. Although the use of diclofenac was recently banned, following conclusive proof supporting the safety of meloxicam, the toxic effect of other veterinary NSAIDs needs to be determined. One manner of determining a drug's safety would be to develop an *in vitro* model. In an attempt to establish this *in vitro* model it was first necessary to establish the mechanism of diclofenac's toxicity. For this purpose three hypotheses were proposed and tested: vasoconstriction of the renal portal veins with subsequent necrosis; increased production of reaction oxygen species (ROS) with subsequent activation of the mitochondrial apoptotic cycle; and a decrease in the secretion of uric acid by renal tubular epithelial (RTE) cells. To test these hypotheses *ex-vivo* tissue models were established using tissue from either the domestic chicken (*Gallus domesticus*) or the African White-backed vulture (*Gyps africanus*), both previously validated as models[5,6]. We demonstrated that both diclofenac and meloxicam are directly toxic to the chicken and vulture renal tubular epithelial (RTE) cells following 48h of incubation. It was shown that toxicity was associated with an increased production of reactive oxygen species (ROS), which could be temporarily ameliorated by pre-incubation with uric acid (UA) due to its anti-oxidant activity. When cultures were incubated with either drug for only two hours, meloxicam showed no toxicity in contrast to the cellular toxicity present for diclofenac. In both cases no increase in ROS production was evident. In addition both drugs influenced the excretion of uric acid by interfering with p-amino-hippuric acid (PAH) channels. The effect on uric acid excretion persisted after the removal of the diclofenac but not meloxicam. We therefore concluded that vulture susceptibility to diclofenac toxicity resulted from a combination of an increase in cellular ROS, a depletion of intracellular uric acid concentration, an important anti-oxidant, and most importantly the pharmacokinetics profile of the drug in the plasma.

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[5] Private Bag X04, Section of Pharmacology, Faculty of Veterinary Science, Onderstepoort, 0110. vinny.naidoo@up.ac.za. +2712 529 8368

The last decade has seen the catastrophic decline in the vulture population on the Asian subcontinent following their inadvertent exposure to diclofenac. Diclofenac toxicity in vultures being associated with only severe visceral gout on necropsy has lead to the speculation that the kidneys are the site of drug toxicity[7]. Meteyer *et al.* proposed that the inhibition of renal prostaglandins (PG) and subsequent closure of the renal portal valves was a cause of renal ischemia[18]. Although current literature does not support this proposal as the closing of the reanl portal valve in birds promotes renal perfusion it is still possible that diclofenac interferes with vascular tone of the renal portal vascular network[8,9]. Ng *et al.* (2006) proposed that the DF toxicity results from ROS induced oxidative damage to the mitochondria and subsequent activation of the apoptotic cycle[10]. To test these hypotheses in avian models, organ baths with renal vasculature tissue or primary cell cultures, using tissue from the validated *G. domesticus* model, were set up. In all cases the toxicity of DF was compared to meloxicam (MLX) and related to the pharmacokinetics of each drugs.

In addition to the above hypotheses, we futher proposed that DF could be toxic due to the inhibition of UA excretion. Studies by Naidoo *et al.* (2007), demonstrated that the clinical signs of depression, following exposure to diclofenac, were a direct result of the marked increase in plasma uric acid concentrations[5]. Since the mammalian kidney is influenced by the uricosuric effects of DF and MLX through the inhibition of the basolateral UA-excreting organic anionic transporters (OATs) (also referred to as a PAH transporter), multi-drug resistance protein (MRP) and the apical UA reabsorptive channel known as URAT1, it is possible that a similar effect occurs in the vulture. This is supported by similar PAH and MRP channels that are involved with UA excretion in the chicken (avians do not reabsorbed UA) as well as by both diclofenac and phenylbutazone been known to induce uricemia in a validated chicken model[11,12 5,13-15]. To test this hypothesis an artificial renal tubule was set up using either chicken or vulture RTE cells. The results from the culture assay were confirmed *in vivo*.

In the organ bath studies neither MLX (n=2) nor DF (n=4, $p = 0.028$) increased the contractile responsiveness of the venous smooth muscle to NE (**Figure 7-1**) i.e. they promoted vasodilatation in an irreversible manner opposing the hypothesis by Meteyer *et al.* (2005). This can be explained by the finding of Peredo (2003) who demonstrated that

indomethacin inhibited renal vasoconstriction by inhibiting contractile PG$f_2\alpha$ production via cyclco-oxygenase (COX) inhibition[5]. Both DF and MLX are COX inhibitors. The effect of COX inhibition lasting for up to 12h, will therefore explain the irreversible effect seen.

In chicken RTE cell cultures DF (n=9) and MLX (n=9) were cytotoxic in a time and concentration dependent manner (Figure 7-2) and the effect was associated with ROS formation. ROS peaked at 12h (>200%), corresponding to the first signs of toxicity seen with 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT). This supports the view that that toxicity was related to mitochondrial death (apoptosis) induced by ROS formation as proposed by Ng *et al.*.

Exposure of RTE cells to the toxic effects of DF for 12h does not take into account the its short half-life of 0.6h in the chicken i.e. chicken toxicity was not related to long-term exposure.[6] In the case of MLX, vultures were not exposed to plasma concentrations above 5ug/ml for more than 0.5h[16,17]. To accommodate these pharmacokinetic features RTE cells were subsequently exposed to DF (n=9) and MLX (n=9) for only two hours. In this assay no toxicity was seen for MLX, while DF remained significantly toxic ($p < 0.05$ for all concentrations). DF toxicity was not associated with ROS production, suggesting an additional mechanism of toxicity.

To determine whether reduction of intracellular UA concentrations was involved in the mechanism of DF toxicity cultures were incubated concurrently with UA and drug for up to 48h (n=9). In all cases cell survival was promoted for 24h which corresponds to the first *in vivo* signs of toxicity in poisoned birds (Figure 7-2)[18]. This is due to the intracellular anti-oxidant effect of UA that would buffer the ROS induced by the drug. However, the short duration of protection was unexpected tending to suggest that the cell was somehow either being depleted of UA within 24hours or UA was being neutralised. However, the results support our hypothesis that toxicity could be related to the inhibition of PAH and MRP transporters and alteration in UA excretion.

To confirm the influence of DF and MLX on UA transport, an artificial renal tubule using chicken RTE cells was set up using PAH, a specific indicator of UA transport by OAT

channels. All three concentrations of DF (n=4) decreased PAH excretion with the highest dose being the most significant (p = 0.006) (Figure 2-9). Although meloxicam tended to influence PAH clearance, the effect was not significantly different to the control (p=0.3). These results indicate that DF could reduce UA clearance by inhibiting PAH transport.

To rule out interspecies differences, the assay was repeated with vulture RTE cells using UA instead of PAH, following two hours of exposure. All three concentrations of DF (n=2) tested, promoted a concentration dependant decrease in UA clearance. In contrast no significant effect was noted with MLX (n=2) (Figure 7-4). This once again confirms that DF decreases UA excretion in the kidney.

With the *in vitro* effect being so prominent, the *in vivo* effect of MLX and DF on UA transport was subsequently conclusively confirmed in vultures (n=4 for each drug)(Figure 7-3), where MLX inhibited UA excretion for the short duration of 1.5h while DF continued to induce a significant increase in plasma UA from pre-treatment concentrations (P = 0.037). For both MLX and DF, the plasma UA concentrations did not signifianctly differ between the two drugs in the first 1.5hrs. The effect corresponded with the half-life of the respective drugs in the vulture i.e. the effect of MLX was only transient and and reversible on drug elimination. This corresponds to the *in vitro* toxicity study, which indicated that toxicity was directly related to the half-life and mean residence time of either of the drugs within the body.

While the increase in vulture plasma UA concentrations could be explained by constant inhibition of the channels brought about by constant drug exposure, the drug was completely excreted from the chicken 8 hours post-exposure, despite signs of toxicity seen 48h later. This pointed to prolonged, most likely irreversible channel inhibition. To ascertain if DF could inhibit transporters after drug withdrawal, the vulture RTE cells were exposed to DF for 5 hours followed by complete flushing of the chambers (n=2), prior to commencing the transporter assay. Similar to the result previously obtained, DF induced an increase in UA concentrations in both the apical and basolateral wells. More importantly the total concentration of UA in each well was greater than that added (Figure 7-4), which indicated that DF depleted the cell of UA. The increase in UA in the apical and basolateral

wells indicated that the inhibition was most likely at the level of the basolateral PAH transporter.

With chickens RTE cells being susceptible to the toxic effect of DF and MLX as the result of the inhibition of the OAT/PAH transporters, it may be concluded that diclofenac interferes with uric acid excretion at the level of the kidney, thereby depriving the cell on an important anti-oxidant. Although this does explain the ability of the drug to induce toxicity in both the chicken and vulture, it fails to explain the greater sensitivity of the vultures. The sensitivity may, however, be explained by the pharmacokinetic half-life of 2 and 14h in the chicken and vulture, respectively and the results from the ROS study, in which exposure to diclofenac for only periods above 12 hours enhanced ROS production. Therefore while general toxicity results from the loss of an intracellular anti-oxidant, the additional production of ROS from the long term exposure to diclofenac enhances the overall oxidative stress experience by the RTE cells i.e. they lose an anti-oxidant while being exposed to greater ROS production. The importance of the drug's half-life in toxicity is highlighted by meloxicam's equivalent toxicity profile when exposure times are extended beyond the drugs *in vivo* mean residence time.

Methods Summary

Broilers (n=10) were used for most of the assays. One non-treatable injured African White-back vulture, was also used. For the organ bath studies the cranial renal portal vein was dissected and suspended in physiological saline solution (PSS) containing calcium. Contractile studies were undertaken using a force transducer myograph (Danish Myo Technology) as previously described. [19,20]

The toxicity assay made use of primary renal tubular epithelial cells harvested and established over 48h as previously described[21,22 22-24 25,26]. For direct toxicity, cells were exposed to DF and MLX in the absence or presence of UA. At 12, 24, 36 and 48h post-exposure culture viability was determined using MTT[21,27,27,28]. For ROS formation, established cultures were incubated with DF or MLX for 6, 8, 12 and 24h (Yu 1998). ROS production was determined using DCFH-DA[18,29]. For the delayed toxic effect, chicken

RTE cells were incubated with either DF or MLX for only 2h, with toxicity and ROS production evaluated as above.

For the transporter assay RTE cells, 500ul (10^6 cells/ml) were seeded onto the anipore membrane culture inserts, in 24 well microtitreplates and fed every three days[19]. After seven days of incubation, media was replaced with the HBSS transport medium with either PAH (chicken RTE) or UA (vulture RTE) as previously described[20,21]. Total drug exposure was for 2 hours. The irreversible toxic effect of DF was established and evaluated 2 and 4h following drug removal (Initial drug exposure was 5h). Uric acid concentration was measured with a Nexet Chemistry Analyser (Alfa Klasserman, Bayer SA) while PAH concentrations was analysed as described[22]. For the *in vivo* change in UA stored serum samples (0, 0.15, 0.5, 1, 1,5 and 2h) from birds treated with intravenous DF (n=4) at 0.8 mg/kg (unpublished) and intramuscular MLX administration (n=4) at 2 mg/kg (Naidoo *et al.*, in press) from *G. corprotheres* were analysed.

7.1 Acknowledgements

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7.2 Electronic Addendum

7.2.1 *Methods*

7.2.1.1 Chemical and Consumables

Diclofenac, Meloxicam, Penicillin, Streptomycin, DCFH-DA, Di-methyl sulphoxide (DMSO), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), trypsin, norepinephrine (NE), NaCl, KCl, $MgSO_4$, $NaHCO_3$, KH_2P0_4 , $CaCl_2$, EDTA, glutamine, sodium $_L$-lactate, sodium pyruvate, Glucose and HEPES, p-amino-hippuric acid (PAH) were obtained from Sigma South Africa. Debulco's modified Eagles's essential medium

with L-glutamine (DMEM), foetal calf serum (FCS), Hanks balanced salt solution (HBSS), phosphate buffered saline (PBS) was obtained from Highveld biologicals South Africa. The 96 and 24 well plates and anipore membrane culture inserts was obtained from NUNC, South Africa.

7.2.1.2 Animals

The Broiler chickens (n=10) used in the study were raised at the Poultry Reference laboratories (Onderstepoort, South Africa) on a commercial broiler diet and water available ad lib. The chickens were euthanized at 5 weeks of age by severing the spinal cord. One African sub-adult White-back vulture of unknown age that required euthanasia due to a severe injury was also used. The vulture had not previously been treated with any analgesic and received only fluid therapy intraperitoneal. Euthanasia was performed using pentobarbitone.

7.2.1.3 Dose Extrapolations

For the organ bath studies, the concentration of diclofenac and meloxicam tested represent the maximum plasma concentration of these drugs in vultures. For the toxicity and transporter assay, the concentrations tested are based on the extrapolated LD_{50} of the diclofenac/meloxicam in the chicken and vulture, depending on which cells were used

7.2.1.4 Organ bath studies

The cranial renal portal vein was dissected away from the parenchyma of the kidney. The vein was suspended in physiological saline solution (PSS) containing calcium. Contractile studies were undertaken as previously described[22]. The dissected vessels were attached onto the force transducer of a myograph (Danish Myo Technology). and were allowed to equilibrate for 60 minutes in oxygenated PSS (5% CO_2 in air). Initial contraction was stimulated with potassium chloride and thereafter with NE (10 μmol) before and after treatment with DF (0.42 μmol) or MLX (0.66 μmol). The reversibility of the contractile response to NE was determined by flushing out the organ chambers thoroughly at least three times, allowing the tissue to equilibrate prior to re-stimulation with NE, without exposing tissue to . DF was tested in quadruplicate and MLX in duplicate due to technical difficulties.

7.2.2 *In vitro RTE assay*

All assays were repeated in triplicate in a plate and were repeated on three different occasions.

7.2.2.1 Establishing of Cell Cultures

From the chickens and the vulture, the kidneys were harvested immediately after euthanasia, homogenised with a scalpel blade and incubated overnight in DMEM with 1mg/ml of collagenase at 37 ºC[23,24]. The following morning the cell homogenate was passed through 250 and 38 micron filters to isolate the RTE cells[25]. The isolated cells were washed three times at 200 x G with PBS and re-suspended in DMEM supplemented with 10% FCS, streptomycin and penicillin G[26].

7.2.2.2 Toxicity Assay

Chicken and vulture RTE cells, 200ul (10^5 cells/ml) were seeded into 96-well plates[27]. The assays were allowed to establish over 48 hours, prior to incubation with the drugs. The chicken and vulture RTE cells were exposed to various concentrations of DF and MLX based on plasma concentrations previously measured in these species. Following predetermined intervals, the cultures were incubated with MTT (0.44 umol) for five hours[21,27]. The degree of formazan formation was determined by rinsing the wells with HBSS prior to the addition of 50µl of DMSO and subsequent reading on a Varion spectrophotometer at 570nm (1cm path length)[28]. For the delayed toxic effect, chicken RTE cells were incubated with either DF or MLX for two hours (2 half-lives in the chicken), flushed with HBSS three times and replaced with fresh medium. Toxicity was evaluated as for the standard toxicity assay. In all cases the degree of cell death, following drug exposure, was evaluated as a percentage of growth of untreated wells on the same plate (% Cell Viability).

7.2.3 *ROS studies*

Chicken RTE cells were grown as for the toxicity assay. The cells were incubated with DF or MLX in the presence or absence of the uric acid for 6, 8 and 12h (Yu 1998). The cell cultures were thereafter incubated with DCFH-DA for 30 minutes and washed with HBSS[29]. The change in absorption was read on a Verion spectrophotometer at 504 nm. The delayed toxic effect was determined using the same culture method as for the toxicity assay. The degree of ROS production was evaluated as a percentage of the ROS production in untreated cells grown on the same plate.

7.2.4 *Transporter Assay*

To ascertain if either DF or MLX had an influence on UA transport, stored serum samples following the intravenous DF (n=4) at 0.8 mg/kg and intramuscular MLX administration (n=4) at 2 mg/kg from *G. corprotheres* were analysed for the change in serum UA concentration in the first two hours following dosing[6]. Uric acid was measured with a Nexet Chemistry Analyser (Alfa Klasserman, Bayer SA).

To determine the *in vitro* influence on UA transport, 500µl of vulture RTE cells (10^6 cells/ml) were seeded onto the anipore membrane culture inserts, in 24 well microplates and fed every three days. After seven days of incubation, the culture medium was replaced with the HBSS transport medium as previously described. The reaction was started by adding the drug into the basal well. Cell integrity was initially established by the addition of inulin into each well. For the standard assay the assays were exposed to various concentrations of DF and MLX following 2 hours of incubation to ascertain their effect on UA excretion. The assay was subsequently repeated with chicken RTE cells with PAH. PAH concentrations were analysed as previously described. Both the UA and PAH transporter assays were repeated in duplicate for each time point. The change in concentration in each well was established as the difference from the start to the stop of incubation. The net excretion was calculated as the change in the basolateral concentration

subtracted from the apical change. For data analysis the UA flux of the control well was subtracted from the drug results to establish the change in UA excretion.

Based on the positive results of the above transporter assay, the delayed toxic effect of DF was determined at the 0.3 uM concentration. Five hours after incubation, the wells were repeatedly flushed and the media replaced with the transport buffer and the change in UA concentration was established at 2 and 4h post-incubation. UA was analysed as above.

7.2.5 *Statistics and Repeatability*

DF in organ baths was repeatable on four occasions. All toxicity assays were assessed in triplicate for each point on three different occasions, while the PAH transporter studies assessed in duplicate on two separate occasions. Vulture assays could not be repeated due to the absence of sufficient organs for harvesting. Results are presented as mean ± the standard error of the mean (SEM). Significance per drug concentration in comparison to the control was assessed using a t-test. For organ baths, PAH transport and *in vivo* change in UA excretion difference over time were compared to pre-drug concentration using paired t-test (SPSS 13, SPSS Inc.).

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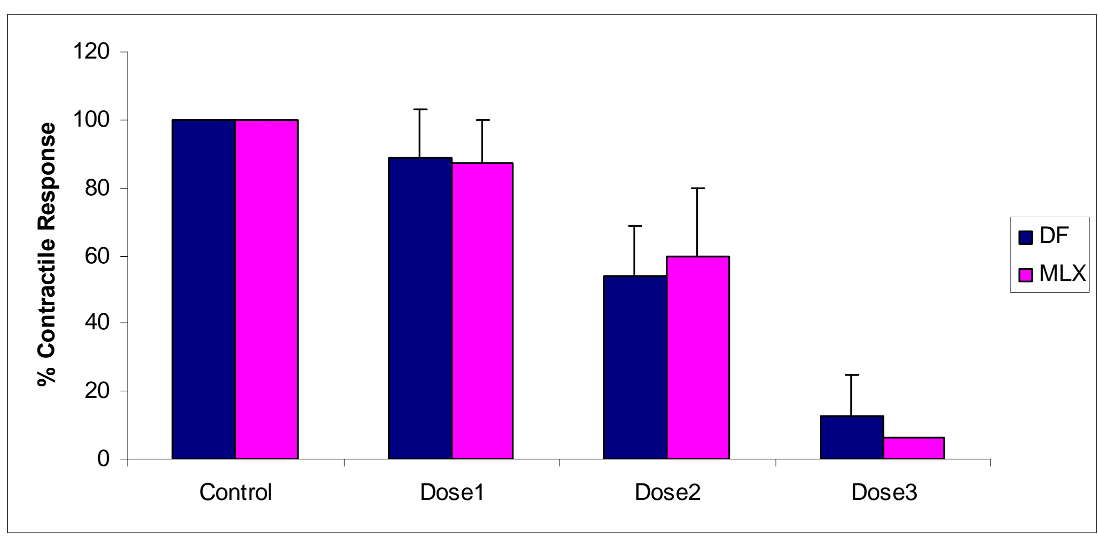

Figure 7-1: Contractile response of chicken cranial renal portal veins to norepinephrine (NE) alone (control) or the response to NE following co- incubated with either diclofenac (DF) or meloxicam (MLX) as a single dose of drug. Dose 1 to Dose 3 illustrates the additive inhibitory effect of the NSAIDs in an irreversible manner, as the chambers were thoroughly flushed prior to each dose. Results are presented as mean ± SEM

a

b

c

Figure 7-2: Direct cell toxicity of DF and MLX following variable periods of incubation and/or concentrations using the MTT assay. Results are presented as mean ± SEM.
a) chicken RTE cell viability following 12 hours of incubation with DF or MLX at four concentrations; b) chicken RTE cell viability following incubation with DF (470nmol) or MLX (13nmol) in the absence and presence of UA over 48 hours [+ UA-indicates that the cultures were incubated concurrently with UA for the assay]; c) vulture cell viability following incubation of DF and MLX for 48h in the presence of UA

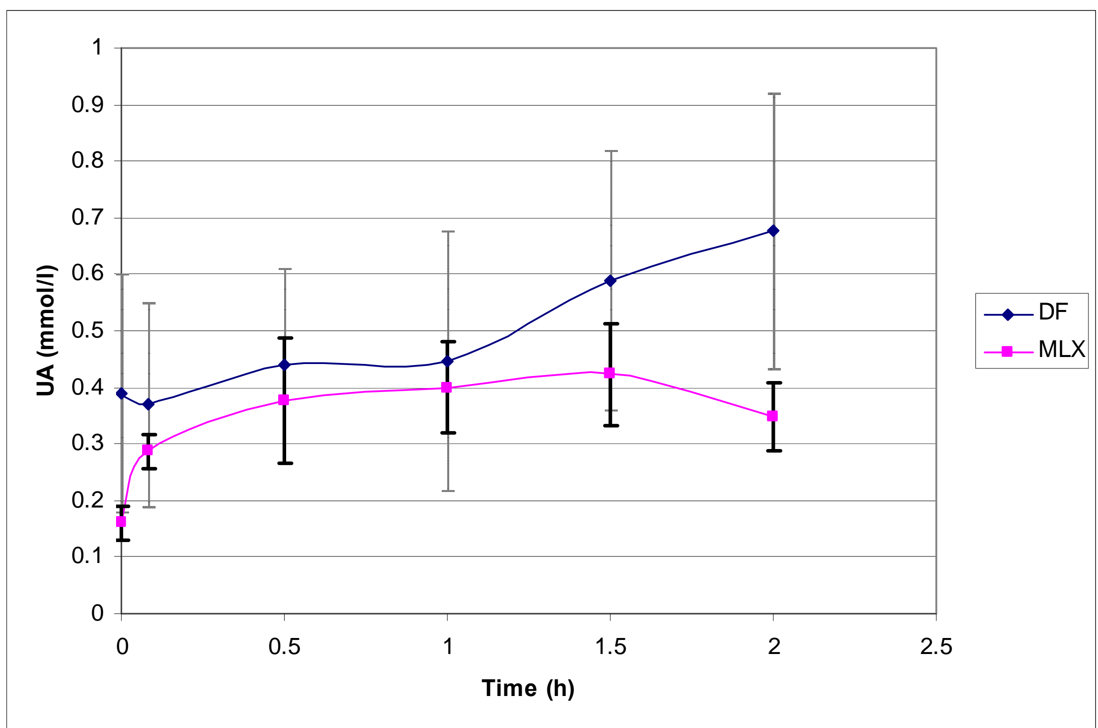

Figure 7-3: *In vivo* change in serum UA concentration for *G. corprotheres* over the first two hours following the administration of MLX (n=4) or DF (n=4)(p=0.037). Results are presented as mean ± SEM

a

Change in PAH concentration (ug)
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
Drug
C, DF 20, DF 40, DF 80, MLX 13, MLX 26, MLX 54

b

Change in UA concentration (umol/l)
0
-0.05
-0.1
-0.15
-0.2
-0.25
-0.3
-0.35
-0.4
0 5 10 15 20 25 30 35 40
Conc (uMol)
DF, MLX

c

Change in UA concentration (umol/l)
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
0 1 2 3 4 5
Time (h)
DF, Control

Figure 7-4: Influence of DF or MLX on UA or PAH clearance in cell cultures established in double chambered well. Results are presented as mean ± SEM.
a) change in PAH concentration for chicken RTE cells following 2 hours of incubation with DF or MLX; b) change in UA concentrations for vulture RTE cells following incubation with DF and MLX for two hours c) Change in UA concentration for vulture RTE cells, 2 and 4 hours following the removal of DF from chamber.

CHAPTER 8: General Discussion

8.1 Hypothesis 1: Meloxicam as a vulture safe alternate

Following an extensive four phased, single dose tolerance study, meloxicam was established as a vulture safe product at a dose of 2mg/kg, which represented the maximum theoretical intake for a bird consuming a 1kg meat meal in a three day cycle. In this study the safety of meloxicam was established as both pure drug in formulation and as parent drug with associated cattle specific metabolites in residue rich cattle liver or muscle. Since the single dose tolerance study reflected the worst case scenario of one large exposure the cumulative toxic effect of a molecule also required consideration. This was especially important as individuals feeding once a day could be exposed to more frequent, albeit lower total doses, which has the potential to reach steady state and/or toxic concentrations. To ascertain the validity of this supposition a pharmacokinetic study was undertaken to establish the half-life of meloxicam and thus it's potential to accumulate.

In this pharmacokinetic study 12 Cape griffon vultures were dosed with meloxicam in a parallel designed study by either the oral or intramuscular route at 2mg/kg. Once again, as for the tolerance study, no signs of toxicity were evident in any of the treated animals. More importantly meloxicam was characterised by a very short half-life of approximately one hour, making it unlikely that this drug could accumulate following single daily exposure even at the very high dose used. Further support for the safety of meloxicam was evident in clinically-managed injured vultures at a daily dose of 2 mg/kg, in periods ranging from five to fourteen days, in which no overt signs of toxicity were seen. The results of safety studies confirm that meloxicam is safe in vultures. Therefore hypothesis 1 can be accepted.

With conclusive proof supporting meloxicam as the safe alternate that could replace diclofenac as the NSAID of choice for use in cattle, the Indian autohority has recently placed a ban on the manufacture and importation of veterinary diclofenac in their country; a resolution that has subsequently been implemented by the Nepali and Pakistani governments. Although this will reduce the environmental concentrations of diclofenac in

the region over the next few years, only time will tell if the ban on the sale of diclofenac was an environmental success. Unfortunately it may be too late to save the wild species as continuous losses of up to 30% a year, have seen the population of Oriental White-backed vultures reach a cumulative decline of up to 99.9%, with the species already extinct in certain parts of India (R Cuthbert, Per Comm, 2007).

8.2 Hypothesis 2: The influence of diclofenac on uric acid excretion

In the initial step in establishing diclofenac's mechanism of toxicity, the acute toxic effect of the drug was evaluated in the domestic fowl in the attempt of validating this easily obtainable species as a surrogate model. Following intramuscular administration at various doses, the injected fowls showed identical clinical signs, time to clinical signs, clinical pathological changes and necropsy changes together with similar histopathological lesions albeit more severe in the chicken. A major difference was, however, present in the LD_{50} as the fowl was approximately twenty-fold less sensitive. Additionally the pharmacokinetic profiles were very different with the half-life in the fowl being substantially shorter. Unfortunately with the pharmacokinetics and LD_{50} being markedly different, the domestic fowl could not be used as a surrogate in establishing the safety of other NSAIDs as they will not be sensitive indicators. However, since the clinical and necropsy findings were almost identical the domestic chicken was used in subsequent studies aimed at establishing the mechanism of drug toxicity.

One other finding in the domestic fowl of a terminal increase in serum potassium concentrations prior to death was later also confirmed in the vulture following the analysis of banked samples. Although the potassium concentrations from the vulture appeared high, their true significance could not be evaluated as normal reference intervals were not available for the species. To allow for proper clinical evaluation the references for selected clinical pathological parameters were established allowing the increase in potassium to be properly evaluated. From the two birds that died following the administration of diclofenac, the potassium concentrations were 29 and 30 mmol/l at the time of death, making this twenty fold higher than the mean for the species.

An additional finding in the domestic fowl was a terminal drop in pH. Although this could not be confirmed in the vulture, due to the absence of fresh blood to perform a blood gas analysis, this finding is still considered to be very important. In addition to explaining the increase in serum potassium, the acidosis could explain the clinical sign of depression evident in the birds as acidosis is known to induce depression in various animal species. More importantly, with the onset of clinical signs of toxicity being associated with an increase in uric acid and uric acid having the ability to induce an acidaemia, a causal relationship between the uricaemia and the clinical signs exists and therefore provides the sequence in which toxicity develops i.e. the drug toxicity promotes the decrease in uric acid excretion either directly or indirectly, which subsequently promotes the development of the clinical signs of toxicity.

With the chicken being a tested model for establishing the mechanism of toxicity, three theories were evaluated. In the process of evaluating the theory by Meteyer *et al.*. (2005), an extensive literature review showed inconsistencies in the theory proposed as the valve functions physiologically in an opposite manner to that proposed *viz.* it is the open valve that predisposes the kidney to toxicity and not a closed valve as proposed. However with a slight modification to the theory, the site of drug toxicity could possibly lie at the level of the renal portal vein and not its valve. With the renal portal vein providing up to 75% of the blood supply to the avian kidney, it is possible that prolonged venoconstriction in these vessels could induce ischemia of adequate severity to induce necrosis of the renal proximal convoluted tubules. With the NSAIDs being known inducers of vessel contractility, through the inhibition of vasodilative prostaglandin E_2 synthesis, the level of inhibition would therefore most likely lie at the level of the COX enzyme.

To ascertain if the modified theory had merit, isolated cranial renal portal veins were incubated under isometric conditions in organ tissue chambers. Once basal stimulations with norepinephrine were established, the co-administration of either diclofenac or meloxicam to the chambers produced an antagonistic effect on the activity of all subsequently added norepinephrine i.e. it reduced the contractility of the vascular tissue. This induced effect was also irreversible as the response to further doses of norepinephrine was blunted despite washout of all NSAIDs from the chambers. From this result it can be concluded that contractility of renal vasculature is not a factor in the toxicity of diclofenac.

Although the exact mechanism behind the decrease in contractility is unknown, the effect may be explained by previous findings that demonstrated the importance of PG*f*α in certain vascular beds of the kidney. In these specific vascular beds PG*f*α mediates vascular contractility such that inhibition of the COX enzyme decreases PG*f*α content thereby promoting vasodilation.

The second theory advanced by Ng *et al.*. was the production of ROS as the mechanism renal cellular toxicity. They supported their theory with studies using mammalian tissue. Although the mechanism has merit, no evidence was provided to support this interspecies supposition as no comparative avian studies were undertaken. To therefore allow for a more avian specific model, primary cell cultures were established from the fowl kidney. As proposed by Ng *et al.*, diclofenac was shown to be toxic as a result of the production of ROS. Unfortunately the importance of the result was questionable as meloxicam also proved to be just as toxic from the production of ROS. With the production of ROS peaking 12 hours after incubation with drug, it was speculated that the pharmacokinetics of the molecule could be important, as cellular exposure *in vivo* to either meloxicam or diclofenac was unlikely to extend to 12 hours i.e. both drugs are characterised by an extremely short half-life in the fowl.

To accommodate for the pharmacokinetics of the molecules a PK/PD link was established by exposing culture to either diclofenac or meloxicam for approximately five diclofenac half-lives followed by the flushing of drugs from the wells. As expected from the *in vivo* toxicity studies, meloxicam was non-toxic while diclofenac remained toxic, thereby illustrating the importance of the drug's half-life in the mechanism of toxicity. While diclofenac was clearly toxic in a delayed manner, toxicity was not associated with the formation of ROS only, thereby suggesting that some additional mechanism was associated with the drug's toxicity. This study also provided information on the molecular aspects of toxicity, viz. the ability of diclofenac to be toxic in a delayed manner could only result from the prolonged inhibition of cellular process.

Although all the available information tended to suggest that theories of Ng *et al.*. (2006), was incorrect, one aspect of the *in vitro* model appeared inaccurate, as no uric acid was added into any of the culture medium despite its prominence in the *in vivo* toxicity of the

drug. To accommodate for this short-coming uric acid was subsequently added into the culture medium concentrations approximating those in plasma and the toxicity assays were repeated. An interesting and unexpected result was evident for the first twelve hours as no cell death was evident in the uric acid wells, while the control well incubated with only diclofenac already showed evidence of toxicity. To understand the importance of uric acid to the cell, its physiological role in protecting the cell from the naturally produced ROS must be considered viz. with the initial ROS assay demonstrating cell death from the production of ROS at 12 hours, it may be concluded that uric acid was providing an important anti-oxidant effect within the cell in the first 12 hours.

While the reason for uric acid's short lasting protection is unknown, it was speculated that the effect could result from drug induced depletion of uric acid from the intracellular environment, i.e. theory three. To ascertain if either diclofenac or meloxicam had an influence on uric acid transcellular movement, isolated fowl RTE cell cultures were established in double chambered wells as a simulation of a functional proximal convoluted tubule. Using PAH, a specific molecule marker for uric acid excretion, diclofenac significantly reduced the cellular excretion of uric acid. To ensure that the effect seen in using the chicken culture was representative for the vulture as well, limited studies were undertaken using cells isolated from one vulture.

A similar effect was also evident using the vulture cultures even though the assay employed was modified with uric acid movement being directly evaluated. When the cultures were incubated with diclofenac for only a few hours, followed by the complete flushing of the wells, diclofenac continued to influence the excretion of uric acid. More interesting, the amount of uric acid recovered was greater than placed in, thereby leading to the supposition that diclofenac interferes with uric acid transport to such an extent that the cell is finally depleted of intracellular uric acid. To confirm the *in vitro* result, the *in vivo* changes in uric acid plasma concentrations in the first two hours following dosing with meloxicam or diclofenac were evaluated. As seen with the *in vitro* assay, diclofenac induced a significant time dependant decrease in uric acid plasma clearance that was significantly different to pre-treatment levels. In contrast meloxicam induced a minor transient change in uric acid concentration with the 2 hour post-dosing concentration reaching pre-treatment levels. We therefore postulate that the diclofenac inhibits the

secretion of uric acid and in the process depletes the cell on uric acid and an important intracellular anti-oxidant, thereby allowing hypothesis 2 to be accepted.

It may be therefore concluded that diclofenac interferes with uric acid excretion at the level of the kidney, thereby depriving the cell on an important anti-oxidant. Although this does explain the ability of the drug to induce toxicity in both the chicken and vulture, it fails to explain the greater sensitivity of the vultures. The sensitivity can, however, be explained by the pharmacokinetic half-life of 2 and 14h in the chicken and vulture respectively and the results from the ROS study, in which exposure to diclofenac for only periods greater than 12 hours enhanced ROS production. Therefore while general toxicity results from the loss of an intracellular anti-oxidant, the additional production of ROS from the long term exposure to diclofenac enhances the overall oxidative stress experience by the RTE cells i.e. they lose an anti-oxidant while being exposed to greater ROS production. The importance of the pharmacokinetic half-life in toxicity is also evident with meloxicam, which became non-toxic when cells were exposed for a period equivalent to the drugs' half-life. This unfortunately limits the usefulness on an *in vitro* model as the pharmacokinetics of the molecule needs to be established first.

8.3 Further studies

Although meloxicam has been shown to be an adequately safe in the vulture, the safety of other veterinary NSAIDs still needs to be determined. With the pharmacokinetics of the molecular being important in drug toxicity, it is important that the half-lives of the other NSAIDs be established, so that their toxicicity can also be evaluated using the above cell culture assays. While this could mean more animal phase testing, it may be possible that *in vitro* methods, such as isolated mixed function oxidases, may also be of value.

Although the use of primary cell culture proved to be effective, the system is very reliant on constant harvesting of tissue samples and therefore ethically questionable. Once manner to overcome this shortcoming would be to sequence and clone the OAT transporters present in the avian kidney. Using established methods, it may thereafter be possible to clone the transporter into an established cell line so that more routine screening may be possible.