The insensitivity of the crow (*Corvusalbus*) to diclofenac toxicity.

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2011

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DECLARATION

The experimental work described in this dissertation was conducted in the Section of Pharmacology and Toxicology of the Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, under the supervision of Professor Vinny Naidoo.

I declare that the dissertation hereby submitted to the University of Pretoria for the degree Masters of Science has not been previously submitted by me for a degree at this or any other university that this is my own work except where inputs have been made by others have been duly acknowledged.

_____ _____

Ms K Mompati Prof V Naidoo (Supervisor)

ACKNOWLEDGEMENTS

I would like to give my sincere gratitude to the following

1. God for the love, guidance and mercy, he has shown me throughout my life.
2. My promoter Professor Vinasan Naidoo (Vinny) for his expert supervision, guidance, patience and understanding enabled me to successfully complete this research project.
3. Prof Neil Duncan for his expertise in pathology and assistance in undertaking the necropsies.
4. Ms Annette Venter and Dr Victor Bagla in assisting with *in vitro* cell toxicity testing.
5. Ms Kerri Wolter for assistance with management of the crows during the study.
6. Dr. Mark Taggart for assisting with drug analysis.
7. The NRF and the RSPB for their financial assistance that made this project successful.
8. To the Department of Health, and my managers, I would like to appreciate and thank them for the time they made available while I completed studies.
9. My family for always giving their unceasing support.

ABSTRACT

Diclofenac has previously been shown to be toxic in three species of *Gyps* vultures (*G. bengalensis, G. tenuirostris,* and *G. indicus*) on the Indian subcontinent. Due to the devastating effect on the population of vultures (>99.9% species mortality), numerous efforts were initiated in order to protect the species. One such effort involved the removal of further threats to the species. At present the major threat identified has been the other non-steroidal drugs (NSAIDs) available for veterinary use. From research on ketoprofen and meloxicam (the former toxic and the latter safe), it was evident that toxicity was not general for the class of NSAIDs and that other factors played a role in toxicity. This unfortunately meant that each drug had to be tested individually in the vulture. While possible, the endangered status of vulture globally makes this approach unethical. As a result an alternate method of testing needed to be validated or sought. It was believed that a surrogate model could be the answer. The aim of this study was to establish if the crow could serve as such a surrogate model.

The toxic effect of diclofenac in crows (n=6) was evaluated in a two cross over studies at doses of 0.8 and 10 mg/kg. No signs of toxicity were evident during the period of clinical monitoring, or necropsy or clinical pathology. In addition the drug was barely detectable in the birds and was described by a half-life of elimination of approximately 2.5 hours. To better explain the absence of observable toxicity, a follow-up study was initiated using freshly harvested renal tubular epithelial (RTE) cells and hepatocytes in a cell culture assay previously validated for cytotoxicity and reactive-oxidative generation. In general, the *in vitro* study results showed the hepatocytes and RTE cells to be tolerant to the presence of diclofenac, with cell viability remaining in the region of 80%. In contrast meloxicam appeared to be more toxic as previously seen with chicken primary RTE cells. Based on the in vivo and in vitro culture results, it was speculated that the absence of toxicity in the crow was due to a combination of rapid half-life of metabolism in combination with low susceptibility of the cells to toxicity.

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To further explain the role of metabolism in toxicity, meta-analysis of pharmacokinetic data for the domestic chicken (*Gallus gallus*), African White-backed (*Gyps africanus*), Cape Griffon (*Gyps coprotheres*) and Turkey vultures (*Cathartes aura*) were evaluated for trends in toxicity. The data clearly showed a trend toward toxicity when the half-life of elimination increased. It was therefore concluded that toxicity in *Gyps* species is probably related to zero-order metabolism, and therefore cannot be predicted by a surrogate model due to inter-species differences in metabolism. The crow is therefore not a surrogate model for toxicity testing in the place of the vulture.

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ABBREVIATIONS

AA	Arachidonic Acid
ALB	Albumin
ALT	Alanine amino transferase
AWBV	African White Backed Vulture
AST	Aspartate amino transferase
CK	Creatine kinase
COX	Cyclooxygenase
CYP- 450	Cytochrome P 450
DCFH-DA	Dichlorofluorescein diacetate
DMEM	Dulbecco's Modified Eagle's Medium
FCS	Foetal calf serum
HPTETE	Hydroperoxy Eicosatetranoic Acid
HETE	Hydroxyeicosatetraenoic Acid
IBA	Important Bird Areas
IUCN	International Union for Conservation of Nature
LTB	Leukotrienes
LD	Lethal dose
LPS	Lipopolysaccharide
MLX	Meloxicam

MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NSAIDS	Nonsteroidal Anti Inflammatory Drugs
OWBV	Oriental White Backed Vulture
PGG	Prostaglandin G
PLA_2	Phospholipase A_2
PGHS	Prostaglandin H Synthase
ROS	Reactive Oxygen Species
RTE	Renal tubular epithelial cells
RSPB	Royal Society for the Protection of Birds
SD	Standard deviation
TXA_2	Thromboxane
UA	Uric acid
WWF	Worldwide Fund for Nature

CHAPTER

1

1. INTRODUCTION

1.1. Introduction

Over the years, the use of the non-steroidal anti-inflammatory drug, diclofenac, in livestock on the Asian subcontinent has accidentally resulted in the dramatic decline in vulture populations via the contamination of their food source. Although the resultant decimation was noticed in the 1990's, it was not until 2004 that Oaks *et al.*, (2004), linked the deaths in the Oriental White-backed vulture (OWBV) (*Gyps bengalensis*), to veterinary use of diclofenac. In a later study, Swan *et al.*, (2006a) were able to show that the African White-backed vulture (AWBV) (*G. Africanus*) was also susceptible to the toxic effects of diclofenac.

In an attempt to protect the remaining vultures numerous efforts were put into place by conservation and governmental organisations. The first of this was the establishment of captive breeding population on the Indian subcontinent to prevent the species from becoming extinct (Buvanendran,*et al.,* 2003). Through a multi-national collaborative research initiative diclofenac was banned by all government on the Indian subcontinent in favour of the safer meloxicam (Virani, 2006). Recently, after Naidoo *et al.*, (2009) demonstrated that the endangered Cape vulture (*G. coprotheres*) was also susceptible to diclofenac, the Medicines Control Council of South Africa issued a pre-emptive ban on the veterinary use of diclofenac in SA.

Although diclofenac was banned across the Indian subcontinent and in South Africa, the hidden risk of other NSAIDs needed to be determined as they could also pose a risk to the remaining vulture populations, if per chance they happen to enter the vulture food chain. In a study conducted by Avise *et al.*, (1988), it was shown that flunixin, another NSAID, was toxic to other raptors such as storks, cranes and owls and even a Marabou stork

(Leptoptiluscrumeniferus). In a study by Naidoo *et al.*, (2010), ketoprofen also proved to be fatal to both the Cape and the AWBV.

As a result the remaining NSAIDs such as tolfenamic acid, meclofenamic acid and ibuprofen now also require testing to ascertain if they possess the potential to be toxic to vultures. Unfortunately with most vulture species being endangered, the *in vivo* testing of these drugs for their safety/toxicity in a vulture model is no longer feasible. As a result it is necessary to find other species closely related to vultures that can be used for toxicity testing. The domestic chicken was initially considered to be the perfect choice (Naidoo *et al.*, 2007). However, attempts to validate the domestic fowl as a model showed the chicken to be less sensitive to the effects of diclofenac and thus an unsuitable surrogate model. More over diclofenac was characterised by a rapid half-life of less than 4 hrs, which was significantly shorter than the half-life of between 11 to 22 hours for vultures (Naidoo *et al.*, 2007).

For this study we attempt to determine the sensitivity of wild caught crows to the effects of diclofenac. The Pied Crow (*Corvus albus*) was selected as the species for evaluation as they are an abundant and sometimes even a pest species in Southern Africa. Crows are also carrion eating animal, which may make them more susceptible than the chicken, which is predominantly a grain eater.

1.2 Hypothesis

Diclofenac is characterised by a prolonged elimination half-life and a low LD_{50} in the Pied crow.

1.3 Aims and Objectives

To validate the crow as the model for toxicity testing of NSAIDs

To gain a better understanding of the mechanism behind diclofenac toxicity

CHAPTER

2

2. LITERATURE REVIEW

2.1. Introduction

Vultures in general, are large carrion eating birds with hooked beaks, featherless heads and soaring food searching behaviour (Avise *et al.*, 1994). They are graceful in the air and awkward on the ground, they soar to rising heights over wide ranging territory and use their sharp eyes to spot any sign of carcasses far below. They can be regarded as supreme scavengers and are as much a part of the wilderness as the giraffe or zebra. In addition to their messy feeding habits, a sinuous neck and bald head is another characteristic feature shared by the vulture species commonly referred to as the griffons.

Vultures have, through the ages, been featured in various different cultures. To some they are "raw necked ungainly ghouls" that are condemned for representing "sloth, filth and voraciousness". The great author Charles Darwin called them "disgusting birds" that "wallow in putridity" (Byrd, 2003). With this said, they have been recognized for their usefulness throughout history viz. In Ancient Egypt the vulture held a very high place of honour as they were a symbol of "purification, compassion, and maternity (Seller, 1992). People revered the bird for its ability to rid them of their carrion (Byrd, 2003).

The goddess Nekhbet, the vulture god, was also the protective deity of southern Egypt, with the result that the vulture was the symbol of the upper kingdom of Egypt and a mark of the pharaohs who represented the highest authority in Egypt (Seller, 1992). In paintings Nekhbet (the vulture) is often depicted flying protectively above the god-king pharaoh, with wings spread open and is best seen on the crown worn by the pharaoh (Fig 2.1). The vulture also being the highest flier was considered to be close to the sun god Ra, which was something that the Egyptians strived for (Byrd, 2003).

Figure 2.1: Nekbet, the vulture goddess on the double crown that was found in the tomb of Pharaoh Tutankhamen (Wiki media, 2002).

Needless to say, vultures are still an integral part of human myth, superstition and culture even in current times. In the western world, vultures are commonly associated with death, a problem which is best highlighted in the Far-side cartoons (Paolillo, 1998). In Africa, vultures are more valued dead, as their appendages are believed to bring good luck (Koenig, 2006). In India specifically, vultures have for centuries been icons of devotion, art, and culture, and have prospered for millennia throughout the region (Virani, 2006) e.g. the vulture god "Jatayu" plays a significant role in Hindu mythology, as Jatayu is said to have given up his life to protect Sita the wife of Rama in the Hindu text The Ramayana (Markandya *et al.*, 2006).

Figure 2.2: Oriental white-backed vultures (*Gyps bengalensis*) feeding on the carcass of a buffalo (R.Risebrough, 2004).

2.2. *The Asian Vulture Crisis*

2.2.1. *Introduction*

While little is known of the life history of vultures, their plight has received a lot of attention recently due to declines in their numbers world-wide (Satheesen and Satheesen 2000; Pain *et al.*, 2003; Naidoo *et al.,* 2009). Nonetheless, this problem has been most marked on the Asian subcontinent, where a major catastrophe was first identified in the late 1980's as three species of vultures (*Gyps bengalensis,*Oriental White-backed Vulture(Fig 2.2 and 2.3 C); *G. indicus,*Long-billed Vulture(Fig 2.3 B);*G. tenuirostris*Slender-billed vulture (Fig 2.3 A) were seen to be declining at an unprecedented rate (Prakash *et al.*, 2003; Oaks *et al.*, 2004; Green *et al.*, 2004). It was eventually shown their deaths resulted through inadvertent, secondary poisoning that occurred from their exposure to residues of the veterinary drug diclofenac in the carcasses they fed upon (Oaks *et al.,* 2004; Green *et al.,* 2004). In 2004 the three species were listed by the IUCN, world conservation union, as critically endangered (IUCN, 2011).

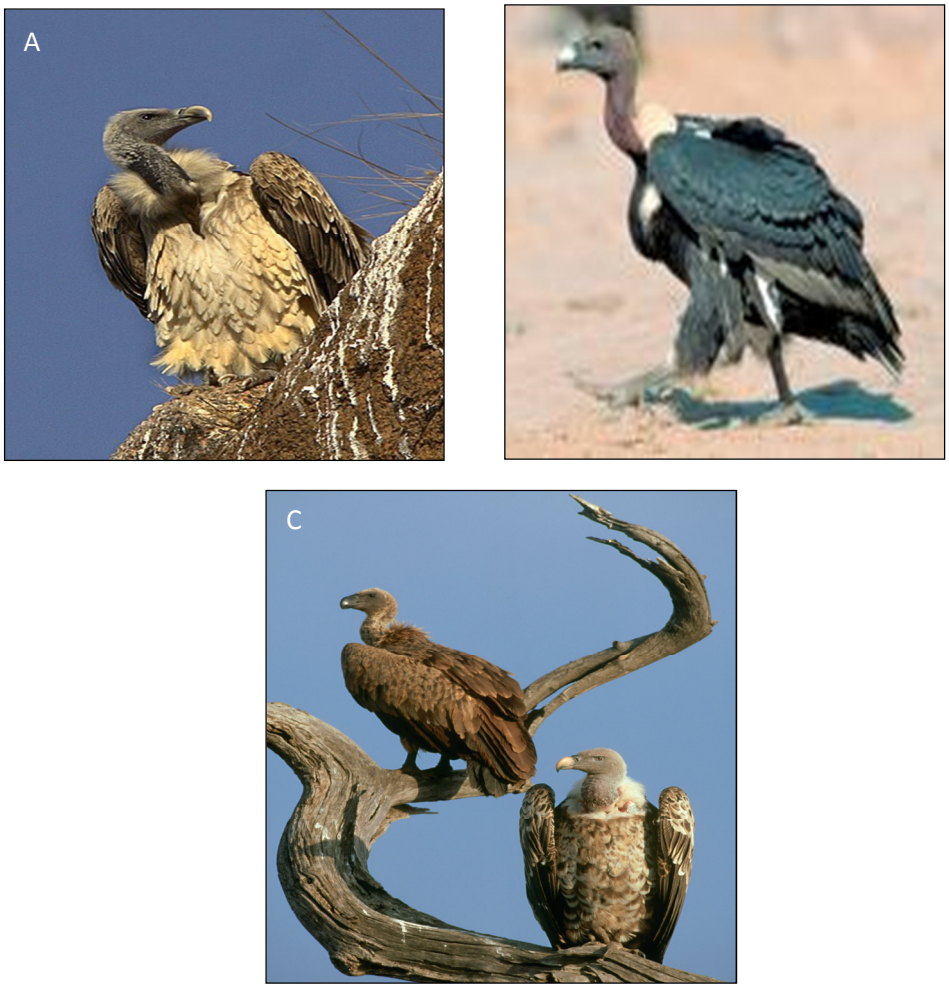


Figure 2.3: Picture of ***Gyps tenuirostris***(A)***,*** *G. indicus*(B), and ***G bengalensis*** (C) in their natural environment (Wiki media, 2007)

The pathology and clinical signs caused by diclofenac in the vulture populations on the Asian subcontinent has also been well documented. In field studies, the sick birds were described as being depressed and literally falling over dead from their nest. The post-mortem findings were also typical, in that there was always severe kidney damage with signs of diffuse visceral gout (Fig2.4) (Oaks *et al.,* 2004; Meteyer *et al.*, 2006; Proffit and Bagla, 2004). In a controlled study undertaken by Swan *et al.,* (2006a) in the African White-backed vulture and later replicated in the Cape Griffon vulture by Naidoo *et al.*, (2009) there was also evidence of abnormal enlargement of the kidneys, spleen and liver with concurrent pallor and mottling. In addition all treated birds had urate tophi covering the thoracic and abdominal air sacs, pericardium and myocardial surfaces.

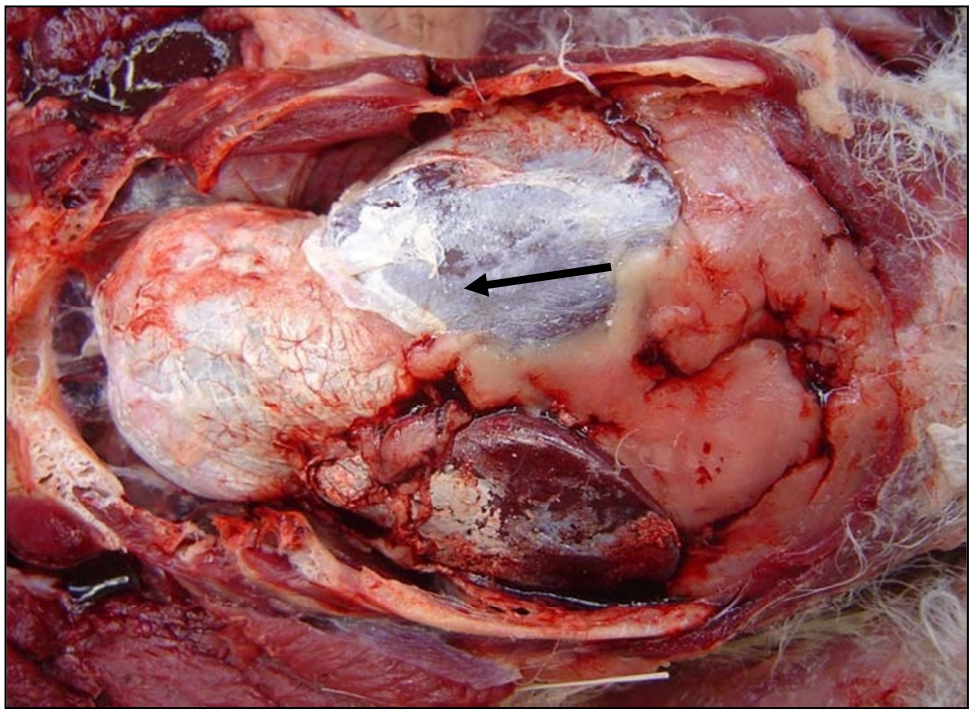


Figure 2.4: Abdominal cavity of vulture (*G. africanus*) at necropsy showing visceral gout (arrow) on the liver surface following experimental exposure to diclofenac. (Oaks et al,2004)

2.2.2. Diclofenac and its accidental contamination of the vulture food-chain

One of the major reasons for the declines in the vulture population was introduction of diclofenac to the Asian subcontinent. To best understand the use of the drug, one first needs to understand the importance of the Non-steroidal anti-inflammatory drugs (NSAIDs) in veterinary science. The NSAIDs are an integral part of veterinary medicine in the treatment of pain and/or inflammation associated with disease especially at the farm level (Fernandez*et al.*, 2007). The latter is important as modern animal welfare practice dictates that animal suffering is unacceptable. Due to the drug's excellent anti-inflammatory properties and cheap price, diclofenac soon became the drug of choice in the management of sick cattle viz. it became the palliative agent of choice in a holy animal (Korom, 2002).

While the NSAIDs, produce a general analgesic effect, they are actually a diverse grouping of chemicals that suppress inflammation through inhibition of one or more steps in the metabolism of arachidonic acid. The group can also be broadly classified into salicylate or carboxylic acid derivatives, indoles, proprionic acid fenamates and oxicams (Boothe, 2001).

2.3. The NSAIDs

2.3.1. Mechanism of action

The prostanoids, prostaglandins, prostacyclin and thromboxane, all metabolites of arachidonic acid (AA), are potent mediators of inflammation in the body. Inflammation is part of the non-specific immune response that occurs in reaction to any type of bodily injury. In the biosynthesis of these prostanoids the first step is the release of AA from the phospholipid membranes through the activation of the enzyme phospholipase A_2 (PLA_2) in response to various stimuli such as cytokine and lipopolysaccharide (LPS) release (Fig.2-5). Arachidonic acid is subsequently metabolized by one of two possible pathways viz, the cyclooxegenase or the lipooxegenase pathway (Burke and Fitzgerald, 2006).The Cyclooxegenase (COX) enzyme is responsible for the generation of the unstable prostaglandin endoperoxide (PGG_2), which through subsequent enzymatic reactions is converted to PGH_2 which is a precursor for all other prostaglandins and thromboxane (Adams *et al*, 2001; Clark, 2006).

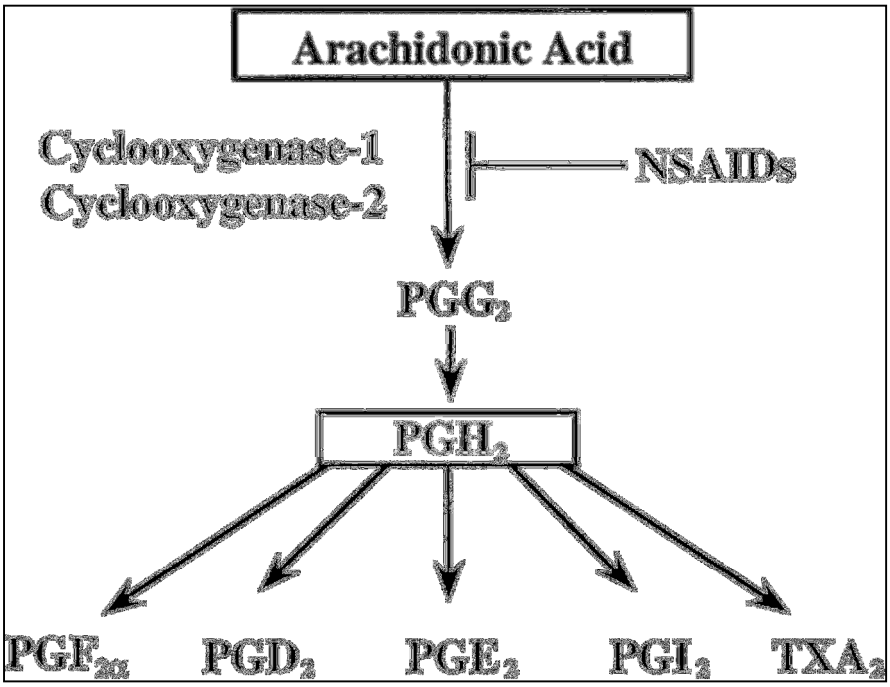


Figure 2.5: The cyclooxygenase pathway responsible for the production of prostaglandin and thromboxane from arachidonic acid (Krakakuer, 2004)

At present three isoforms of the cyclooxygenase enzyme have been identified. The major differences between the COX isoforms lie in the promoter region of the COX genes, the regulation of the mRNA, and the level of their basal expression (Krakauer, 2004). It is believed that the majority of NSAIDs function at the level of conversion of AA to the prostanoids, by inhibiting the cyclooxegenase enzyme system (Krakauer, 2004) (Fig. 2-5).

COX I or prostaglandin endoperoxide H synthases-1 (PGHS1) is protective in nature and mediates formation of constitutive prostaglandins (PGE_2 and $PGI_{2)}$, which are involved in the maintenance of homeostasis especially at the level of the kidney when they are subjected to hypotensive insult. COX 1 also as maintains the integrity of normal gastric mucosa and mediates platelet aggregation (Krakauer, 2004). Inhibition of any of these PGs by the NSAIDs is beneficial in decreasing platelet clotting in case of heart disease. However, inhibition also accounts for toxicity of NSAIDs viz. Inhibition of COX I tends to result in

gastric ulceration, clotting disorders or ischaemic necrosis of the kidneys (Burke *et al.,* 2006 in Goodman and Gillman, 2006).

COX - II is induced predominantly by inflammatory stimuli, such as cytokine release from trauma, immune response or physical injury, and results in the production of PGs. Proinflammatory cytokines include interleukin-1 (IL-1) and tumour necrosis factor (TNFα). TNFα contributes to the pain and swelling of inflammation at the site of injury. COX II is important in ovulation and myometrial contractions during parturition and possibly even renal perfusion (Stanley and Weaver, 1998, Vane and Botting 1987). Inhibition of this COX isoform results in an anti-inflammatory effect while, in general sparing the other constitutive PGs (COX I mediated) involved with homeostasis of organ systems such as the kidney and stomach. Nonetheless some organs still make use of the COX II enzyme as part of their constituent make-up, with the result that its inhibition can result in side effects such as kidney damage (Stanley and Weaver, 1998, Vane and Botting 1987). The selective inhibition of only the COX II isoform in people at least, has been associated with the up-regulation of the COX I isoform with a resultant increase in the chance of fatal thrombo-embolic events (Krakauer, 2004).

COX - III is an isoform that shares all the catalytic and important structural features of the COX I and COX II enzymes. While this isoform is coded by the COX I gene, it differs from the COX I isoform in that it retains an additional nucleotide from intron-1 (Clark, 2006), with the result that it is also referred to as the COX 1a. COX- III is most abundant in the cerebral cortex,and appears to be involved in transmission of painful stimuli from the area at which pain is felt to the central nervous system where pain impulses can be interpreted (Chandersekhar *et al.,* 2002). It has also been implicated in the mechanism of action of paracetamol, which could explain why the drug is effective against inflammatory conditions in the central nervous system, but not peripherally (Chandersekhar *et al.,* 2002).

While the COX enzymes lead to the formation of the prostaglandins from arachidonic acid, the 5 lipo oxygenase (5 LOX) pathway is responsible for formation of leukotrienes. These leukotrienes are important for controlling smooth muscle contraction and are mediators of

inflammation, particularly LB4 which has been associated with recruitment of leukocytes to areas of inflammation (Boothe, 2001). In general, the majority of NSAIDs have no inhibitory activity on the LOX enzyme, with the exception of the newer dual inhibitors such as tepoxalin.

2.3.2. Veterinary use of NSAIDs

Anti-inflammatory: As mentioned above, the effects of prostaglandins include vasodilatation, increased vessel permeability and the chemotaxis of inflammatory cells into the injured region. Through their ability to decrease the formation and thus the quantity of PGs present in tissues, the NSAIDs modulate the degree of the body's normal inflammatory response (Boothe, 2001; Naidoo *et al.,* 2007)

Analgesia: When cells are injured there is a general increase of prostaglandins in injured tissues, which subsequently activate the nociceptor fibres involved in the pain pathway. NSAIDs mediate their effects by decreasing accumulation of inflammatory mediators at the site of injury and are most effective when pain receptors have been sensitized to chemical or mechanical stimuli (Burke *et al.*, 2006). The NSAIDS are generally classified as mild analgesics for the control of acute and chronic musculoskeletal pain.

Anti-pyrexia: The body's set point temperature is mediated in the hypothalamus via the action of the prostaglandins. During conditions characterized by infections and/or inflammation, the PGs in the hypothalamus get up-regulated with a subsequent increase in body temperature. The increased production of PGs is brought about by the COX II enzyme. Aspirin and other NSAIDs inhibit the COX II enzyme and return core body temperature to normal (Burke *et al.*, 2006).

Other beneficial effects: Aspirin, specifically, may be used for its antiplatelet activity in dogs and cats with cardio-vascular disease. This effect is mediated through the irreversible inhibition of platelet cyclooxygenase, and the subsequent decreased production of the eiscanoids which control platelet aggregation (Boothe, 2001). The NSAID piroxicam has been used for the management of cancer in dog, via a yet to be identified mechanism (Boothe, 2001).

2.3.3 Pharmacokinetics of NSAIDs

NSAIDs are usually characterised by good bioavailability from the oral, intramuscular and subcutaneous administration routes (but with possibilities of delayed absorption in horses and ruminants following oral dosing), and demonstrate high plasma protein binding, low volumes of distribution (0.1 to 0.2 L/kg), and limited excretion of the parent drug in urine. They are also characterised by marked inter-species differences in clearance, elimination and penetration into areas of acute inflammatory(Lees *et al.*, 2004).

NSAIDS are metabolized differently in the various species, as a result of differences in the metabolic pathways. This may occur via different cytochrome P-450 (CYP) systems and glucuronidation, or more commonly, via variation in concentration of the same enzyme systems. As such, a drug toxic to one species may have little effect on another, which is particularly important when trying to determine the toxicity. Aspirin for example has a plasma half-life which ranges from 1 hour in ponies up to 37 hours in cats due to the latter's poor glucuronidation ability, while dogs are more susceptible to aspirin's erythrocytic side effects due to their limited acetylation capacity (Boothe, 2001).

While there have been fewer pharmacokinetic studies undertaken in birds, compared to other classes of mammals (Baert,2003) investigated the pharmacokinetics of meloxicam in several bird species and reported different pharmacokinetic parameters being present for each of the bird species tested. It has been suggested that the variability may be due to binding of the drug by unique plasma proteins, which may explain why the volume of distribution of meloxicam was lower in chickens, turkey, and ducks compared to the horse (Baert, 2003).

2.3.4. Adverse reactions in mammal

Despite their common use, the NSAIDs produce a number of undesirable and potentially life threatening side effects. Of these the most commonly seen side effects are those in the gastrointestinal tract such as gastrointestinal ulceration, bleeding and perforation (Lanas, 2001). The mechanism by which gastro-intestinal (GI) damages result is not completely

understood, but it has been suggested that erosion and ulceration reflect inhibition of prostaglandin E_2 and thromboxane A_2 mediated bicarbonate secretion, mucus production, epithelialization and blood flow. As a result the gastric mucus barrier becomes compromised, leading to mucosal damage by the low pH of hydrochloric acid in the stomach. The bleeding has been proposed to result from the breakdown of small blood vessels as a result of a deficiency in mucus in combination with impaired platelet activity (Vane and Botting, 1998; Boothe, 2001).

Renal toxicity is also a common sequelae in humans especially following prolonged use and results from the loss of protective vasodilatory prostaglandins (PgE_2 and PGI_2) which normally ensures medullary vasodilation and urinary output (Boothe, 2001).

2.4 IMPACTS OF THE VULTURE DECLINE

With the vultures being at the top of their food chain, a decline in their numbers has been predicted to have many far reaching economic, ecological, and public health implications (Virani, 2006).

2.4.1. Human health

The decline of the vulture population has led to an obvious (but unquantifiable) increase in the number of putrefying animal carcasses in some areas. Markandya *et al*, 2006 and Pain *et al*, 2003 considered this increase in uneaten carcasses a direct threat to human health as the carrion could provide the perfect breeding ground for pathogenic bacteria. A decline in the numbers of vultures has also resulted in an increase in feral dog population, due to increased availability of food. The feral dog population at one carcass dump increased from around 60 in 1992 to over 1200 in 2001 (Prakash *et al.*, 2007). Feral dog populations could potentially increase the incidence of animal bites and rabies among humans (Prakash *et al.*, 2007).

2.4.2. Costs to Industry

Bone collection for the fertilizer industry has always been a trade amongst the India's poor. Following the effective cleaning of the skeleton of all soft tissues, by the vultures, the bones in the field were picked up for processing. With the decline of the vulture population a number of factors have contributed to a decrease in the available bones for collection viz. an increase in the dog population which consume the bones, the attempted burning of carcasses which destroys the bones and most important the carcasses putrefying in the field (Pain *et al.,* 2003). Indirectly this has burdened the economy, as people who previously were able to sustain themselves through bone collection, are without an income.

2.4.3. Recreation

Vultures have always provided pleasure to bird watchers and in this way contributed to the development of tourism with bird watchers coming from around the world (Markandya *et al.,* 2006). With a decline in their numbers, ecotourism has since dropped.

2.4.4. Cultural and religious values

The decline in vulture numbers has also affected members of the Parsis religion (also known as Zoroastrians) negatively as it has denied them a traditional way of disposing of their dead. The Parsis do not believe in burying or burning their dead as this would contaminate the earth, fire and water which they believe to be pure elements. Zoroastrians also view death as the work of the devil and not of God. As a result they place their dead in areas known as towers of silence, where the cadavers are consumed by the vultures (known as sky burials). Interesting, the Parsis do not attach spiritual value in vultures per se, as the vulture is only a necessary tool in disposal of their dead (Markandya *et al.,* 2006; Koenig, 2006). Therefore the decline in vulture populations has necessitated a change in the "burial" practices of the Parsis, who have now come to rely on strong solar panels to desiccate and hasten the decomposition of their dead. Unfortunately this method of burial is not as efficient as a vulture sky burial (Virani *et al.*, 2006).

2.4.5 Benefits of vulture declines

According to Satheesan and Satheesan (2000) more than 33 aircrafts were destroyed due to collisions with vultures, across the continents of Asia, Africa, Europe and North America, over a period of forty four years (1955-1999). In India alone, about 70 million US dollars were spent on average every year (over a period of 14 years) between 1980 and 1994 to fix airplanes damage by vulture strikes. With the decline in vulture numbers, the number of air strikes has been reduced, resulting in saving by the private and military organizations flying planes over India (Markandya *et al.,* 2006).

2.5. Methods suggested to protect the species

In an effort protect the crumbling population, a number of methods were suggested and implemented by the international community:

2.5.1. Captive Breeding Populations

The establishment of captive populations in India was considered an important step in protecting the species, by maintaining a viable population in captivity. The Royal Society for the Protection of Birds (RSPB) proposed that a minimum of three captive breeding centres be established to hold at least 25 pairs of birds (Watson *et al.,* 2004). The rationale behind this move was to prevent the out-right extinction of the population while maintaining a viable colony under captive conditions. While captive breeding programmes have helped repopulate other endangered bird species (Eames, 2006), this is not considered a viable option for the vulture for the following reasons: Vultures form long-term breeding pairs with the result that there is no guarantee that they will breed in captivity. Pairs usually only produce one egg in a year, and most important the birds only reach breeding maturity at 5-6 years of age or older in some vultures.

The RSPB has since established a number of breeding centres around various parts of India such as Pinjore, Junagadh, Bhopal, Hyderabad and Bhubhaneswar. In 2007, the programme met with onesuccess, when two chicks were successfully hatched at the breeding centre in Pinjore, Haryana. The Birdlife Cambodia Programme has also implemented conservation activities in five priority important bird areas in Nepal (IBAs) namely Boeung Prek Lapouv, Kampong Trach, Stung/Chi Kreng/Kampong Svay, Western Siem Pang Area, and the

Sekong River with the financial support from the Royal Danish Embassy (Danida) and other donor agencies (Eames , 2006).

In 2008 about 88 chicks were hatched successfully from the three breeding centres established in India with 11 chicks hatching at a centre established by WWF in Pakistan, and 14 chicks at the Koshi, Tappu wildlife reserve in Nepal.

2.5.2. Ban on the sale of diclofenac

It was also deemed extremely important that all diclofenac be removed from the market, to prevent further declines of the remaining vulture population. Unfortunately with cattle being a holy species in India, the Indian government was reluctant to issues such a ban until a suitable replacement to diclofenac was identified viz. this new drug needed to not only be vulture safe, but also effective in the management of pain and inflammation in cattle and had to be relatively inexpensive.

In order to identify this safe alternative, the RSPB circulated survey forms to all wildlife veterinarians that had experience in pain management in the vulture (Cutbert *et al.*, 2007). From the survey, the following drugs were identified as potentially toxic; phenylbutazone, caprofen, ibuprofen, and flunixin. In contrast meloxicam and ketoprofen appeared to be safe, with the former being completely safe in more than 60 different species of birds (Swan *et al.*, 2006b).

Following the results of the survey, researchers from a number of different countries undertook studies to determine safety of meloxicam in the African white-backed vulture (G. africanus), which was previously also shown to be sensitive to the effects of diclofenac (Swan *et al.*, 2006a). As a result of this study by Swan *et al.*, (2006b), meloxicam was shown to have none of the side effects typical of diclofenac (Swan *et al.,* 2006b). Following the successful ending on this study, the Indian government finally issued a ban on the manufacture and importation of diclofenac for veterinary use in India in 2006.

2.6. Testing of the other NSAIDs

As a result of the large number of potentially toxic NSAIDs available for veterinary use, it has become important to test as many as possible, for their inherent toxicity. At present the only available method for toxicity testing is whole animal testing, due to the complex physiological process in biochemical action and toxicity. Whole animal testing methods integrate responses across physiologic systems and take into account the susceptibility of different types of tissues (Bucher, 2009).

In addition, toxicity is not always caused by the "parent" compound (the actual chemical to which an animal or human is exposed) but rather to the metabolite of that compound (Bucher, 2009). While *in vitro* or semi-*in vivo* cell culture assays may be useful in predicting toxicity, these cell-based assays might not pick up metabolic or downstream effects. It is also possible that the incorrect cell type is isolated e.g. isolated hepatocytes, as an example, might not reflect toxicity that occurs only in whole liver, where adjoining cells such as biliary epithelial cells may metabolize parent chemicals to toxic forms (Bucher, 2009). It is also possible that different cells show different degrees of sensitivity. The established cell line may lose its differentiation thereby becoming less susceptible to the toxic effects of the drug. In general toxicity testing therefore needs to be undertaken in the species of interest or alternatively in a suitable surrogate. While the studies by Swan *et al.*, (2006) and Naidoo *et al.*, (2009) used South African vulture species for the testing of diclofenac, this is no longer considered a long-term viable option as the Southern African vultures are also becoming endangered.

However, a manner to overcome this short-coming would be to identify another surrogate species. The criteria for this surrogate would be equal susceptibility to the toxic effects of diclofenac as the vulture, be a species that was readily available, and also importantly an easy to work with species. In the first attempt at finding this surrogate, the domestic chicken was tested by Naidoo *et al.*, (2007). Unfortunately, while this study did demonstrate the unsuitability of the chicken as a model viz. the chicken showed rapid metabolism of diclofenac with an elimination half life of less than 4 hours versus the half lives of between 11 to 22 hours in the African White backed vultures at a dose of 0.8 mg/kg. In addition the chicken showed a substantially higher LD_{50} of 10 mg/kg.

As a result of the failure to validate the chicken as a working model, this study was aimed at validating the crow as a model. The crow was selected as the species of interest, as it is commonly available in South Africa, to such an extent that they are considered a pest in some areas. More importantly, the crow shares a number of similarities with the vulture:

- Both species are carrion eaters, while the chicken was an omnivore.
- Both are exposed to similar environments and therefore most likely have similar hepatic metabolic capacity.
- Their levels of uric acid formation and excretion are most likely similar, with the end result being an equal level of stress on the kidneys (Uric acid is a major breakdown product of an all meat diet).

The crow is found mainly in grasslands, open country with trees, clearings, savannahs, riverbanks, and can also be seen near human dwellings, rubbish dumps, and slaughter houses. Crows also patrol for road side kills. It feeds on a number of invertebrates such as insects, spiders and molluscs. It also takes small vertebrates such as rodents, birds, frogs, lizards, fish and bats that can be caught during flight be caught during flight (Richner, 1989)

2.7 CONCLUSION

Due to the toxicity of diclofenac and now ketoprofen in vulture, the safety of the other available veterinary NSAIDs is of concern, and thus need to be evaluated for their toxicity. While in the past this could have been easily facilitated in a vulture, the global endangered status of the vulture population and the large number of NSAIDs that require testing makes such testing very difficult. A surrogate model therefore needs to be found, especially with attempts at validating the chicken as model have proven unsuccessful. Due to the similarity between the crow and the vulture, it is believed that this species may be a suitable surrogate for toxicity testing. The aim of this study will therefore be to investigate the susceptibility of the crow to diclofenac.

CHAPTER

3

3. MATERIALS AND METHODS

3.1. *In vivo toxicity study*

3.1.1 Capture and Housing of animals

Animals were wild-caught in a walk-in baited trap with the assistance of Kerri Wolter of the Vulture Programme of the Rhino and Lion Non-profit Organisation. The necessary permits to catch wild crows were obtained from Gauteng Nature Conservation prior to commencement of the study. The trap used, was made of mesh on three sides plus the top, with the front consisting of pull curtain and a drop-down gate. Once captured, the animals were transported to the Faculty of Veterinary Science, Onderstepoort Pretoria, where they were housed individually in chicken layer cages, at the Poultry Reference Laboratory. The birds were fed with minced meat once daily and had water available *ad lib*. Perches were provided in the cages.

3.1.2. Animal Treatment

The birds were dosed using a two way cross over design. For the first phase birds were dosed with diclofenac (Adco Diclofenac, Adcock Ingram, South Africa) at 0.8 mg/kg (Group A, n=6) or sterile water (Group B, n=6) by gavage. Following a two week wash-out period, the groups were alternated and the birds dosed with either sterile water (Group A, n=6) or diclofenac at 10 mg/kg (Group B, n=6). For the study to continue into the second phase, a prerequisite of no signs of toxicity either clinically or on clinical pathology was applied. No bird received more than a single dose of diclofenac during this study.

3.1.3 Monitoring

Animals were monitored every two hours for the first day and thereafter twice daily until three days post-dosing for clinical signs of toxicity. Blood samples were also collected from

the wing vein in serum tubes for clinical chemistry or heparin tubes for diclofenac analysis. Due to the limited sample volume in the average crow, the birds were sequentially bled (all birds bled before dosing and then sequentially bled at 4, 12, 24 and 48 hours post-dosing) so that the ethical blood volume of 6ml (≤1.5 % of body weight) was not exceeded prior to euthanasia. For clinical pathology the parameters evaluated were sodium (Na^+),potassium (K^+), calcium (Ca^{2+}) (Rapidlab 34E blood gas analyser, Chiron diagnostics, Bayer SA), uric acid (UA), alanine aminotransferase (ALT), aspartate aminotransminase (AST) creatine kinase (CK) and albumin (Alb) (Nexet Chemistry Analyser, Alfa Wasserman, Bayer SA).All clinical pathology analysis was undertaken at the clinical pathology laboratory of the Onderstepoort Veterinary Academic Hospital using commercial analytical methods. Drug analysis was as below.Significant differences in changes in clinical pathology parameters were tested using a univariate analysis of variance (SPSS ver. 19, Chicago, Illinois, USA), with dose and phase used as factors.

Table: 3.1. Bleeding sequence by which the crows were sampled

Time(hrs)	**Control 1**	**Control 2**	**Rx 1**	**Rx 2**
0 (on arrival)	3 birds	3 birds	3 birds	3 birds
4	Bird 1	1	1	1
12	Bird 2	2	2	2
24	Bird 3	3	3	3
48 (Euthanasia)	3 birds all	3 birds	3 birds	3 birds
Total	3	3	3	3

The birds in each group were numbered 1,2 and 3. The listed number represents the specific bird in the group bled at 4, 12 or 24 hours.

Three days after the second phase, the birds were euthanized with a pentobarbitone overdose intravenously and subjected to a full necropsy. Tissue samples collected for diclofenac residue analysis (liver and kidney) were frozen or preserved in buffered formalin.

3.1.4 Drug Analysis

Residue analysis was undertaken by Dr M Taggart of the University of Aberdeen according to the following method: Tissue and plasma samples (0.5 g and 0.3 ml, respectively) were homogenized in 2 ml of acetonitrile (high performance liquid chromatography [HPLC] grade; Sigma-Aldrich, Gillingham, UK) and centrifuged at 1,000 3 G for 10 min to pellet debris. The supernatant was filtered through a 0.2-mm disposable filter unit (13-mm nylon Puradisc; Whatman, Chalfont St. Giles, UK) into a 2-ml crimp-top HPLC vial (Agilent, Stockport, UK). Diclofenac was separated and quantified by HPLC with electrospray ionization mass spectrometry detection (Agilent) 1100 series equipped with a Waters (Elstree, UK) Xterra MS C18 column and equivalent guard. Diclofenac standard (Sigma-Aldrich; D6899) was dissolved into 1:1 (v/v) acetonitrile:Milli-Q (Millipore UK, Ltd., Durham, UK) water at a 50 mg/l concentration. Calibration standards were then created by dilution of this stock standard in 100% acetonitrile to create a calibration range between 5 and 1,000 mg/l. The calibration was linear across this range with an r^2 value .0.99. Samples and standards(20 ml) were subjected to a binary gradient elution profile, which consisted of 0.1% acetic acid (Sigma-Aldrich) in water (solution A) and in 100% acetonitrile (solution B): starting conditions were 75% A to 25% B for 0.1 min, then a 15-min linear gradient was used from 75%-A:25%-B to 5%-A:95%-B, followed by a 5-min column-wash step using 5%-A:95%-B, and finally a 10-min re-equilibration step with 75%-A:25%-B. Flow rate was 0.7 ml/min, and the column temperature was held at 40 C. The mass spectrometer (Agilent; 1946D) was equipped with an electrospray ionization negative ion mode. The mass spectrometer selectively monitored ions 294 and 296 m/z, and 294 m/z (the deprotonated molecular mass) was used to quantify diclofenac residues. The mean diclofenac recovery was 96% in spiked crow plasma and 81% from bovine liver, and the limit of quantification for the method was 10 mg/ l in plasma (Taggart et al., 2009).

3.1.5 Pharmacokinetics Analysis

Due to paucity of the sample collected, as result of the size of the crow (±400g), no complete profiles were obtained or analysable. However, in an attempt to obtain a gross estimate of the average pharmacokinetics of diclofenac in the Pied Crow to allow do a degree of data interpretation, samples collected from individual animals per dose were subjected to naive average pooling, as described in population pharmacokinetics (Sheiner and Beal, 1981).This resultant profile was evaluated using standard equations previously described for sparse data. For interspecies comparison the, standard non-compartmental equations were fitted to the previously published data (Rattner et al., 2008) for Turkey Vultures (*Cathartes aura*;) dosed at either 8 or 25 mg/kg body weight and to sparse data previously presented for a chicken that died when dosed at 5 mg/kg (intramuscular administration; Naidoo et al., 2007; Rattner et al., 2008).

Elimination constant $$\lambda = (\ln \frac{C\ \max}{C\ \min}) /(t2 - t1)$$

Half life $$t_{\frac{1}{2}} = \frac{Ln\ (2)}{\lambda}$$

Volume of Distribution $$Vd = \frac{dose}{C\ \min} * \frac{(1 - e^{-n\lambda t})}{(1 - e^{-\lambda t})} * e^{-\lambda t}$$

Area under curve $$AUC = \frac{dose}{(Vd\ *\ \lambda)}$$

Clearance $$Cl = \frac{dose}{AUC}$$

3.2. INVITRO TOXICITY STUDY

3.2.1 .In vitro toxicity Assay

3.2.1.1 Animals

This part of the study made use of adult crows (n=3) which were caught and immediately euthanized once weekly and subjected to organ harvest. Each crow contributed to a replicate, which was undertaken, 1 per week for 3 weeks. Following euthanasia the kidneys and liver were immediately removed and placed on ice. The organs were subsequently used to ascertain the reason for the insensitivity of the birds to *in vivo* toxicity viz to determine if the renal tubular epithelial and hepatic cells were also insensitive to the toxic effect of diclofenac, or whether the metabolism and absorption were the only factors responsible for the insensitivity evident in the acute toxicity study.

3.3. *Organ Harvesting and culture*

3.3.1. Consumables/ Chemicals

Dulbecco'smodified Eagle's medium (DMEM), Hank Balanced Salt Solution (HBSS), phosphate-buffered saline (PBS), fetal calfserum (FCS) and DMSO were obtained from Highveld Biologicals. The collagenase type II, diclofenac, ,meloxicam, dichlorofluorescein diacetate (DCFH-DA), penicillin G, streptomycin, 3- (4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma South Africa (Johannesburg); culture plates were purchased from NUNC, South Africa (Sandton) and Matrigel was purchased from the Scientific Group, South Africa(Johannesburg).

3.3.2. Kidneys

The kidneys were minced with a scalpel blade and incubated overnight for 24 hrs in Dulbecco's modified Eagle's medium (DMEM) with 0.2 mg/mℓ of collagenase type II at 37 ºC (Freshney 1987). Following this incubation period, cell homogenates were then sequentially passed through a 250 and 38 µm filter to isolate the renal tubular epithelial

(RTE) cells (Naidoo and Swan 2009). The isolated cells were then washed three times with PBS buffer by centrifugation at 200 *g* and subsequently re-suspended in DMEM supplemented with 10% foetal calf serum (FCS), streptomycin (50 IU/ mℓ) and penicillin G (50 IU/ mℓ) (Naidoo and Swan 2009). RTE cell suspensions (200 µl at 10^5 cells/mℓ) were then seeded into 96-well plate (Lu *et al.,* 2004) and given 48 hours at 37 ºC in 5% CO_2 in air to establish, prior to toxicity assay. Adherence was confirmed by visualising the cells under an inverted microscope.

3.3.3. Hepatocytes

Livers were perfused with saline and then with collagenase (0.2 mg/mℓ) via a hepatic vein until the hepatic architecture became disaggregated. The hepatocytes were then collected by incising the intact liver capsule. Isolated cells were washed three times by centrifugation at 200 *g* with PBS buffer and re-suspended using the same media as above. The hepatocyte (200 µl mℓ; 10^5 cells/mℓ) suspension was then seeded into each well of a 96 well plate precoated with matrigel (50µℓ). The 96 well plates were incubated for 48 hours in a CO_2 incubator to establish an adherent culture prior to the toxicity study. Adherence was confirmed by visualising the cells under an inverted microscope.

3.3.4. Toxicity assay with diclofenac and meloxicam: Cell survival and ROS production

Following confirmation of cell adherence, the RTE cells and hepatocytes were incubated with either diclofenac (DF) (at 10, 20, 40 and 80µM concentrations in water) or meloxicam (MLX) at 20,10,5, 2.5 µM for 12, 24, or 48 hours in the presence (DF+UA) or absence DF of uric acid (0.18mM), after a complete media change (same media as described above). Following the pre-determined incubation period, the respectively plates were incubated with 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT; 0.44 µM) for a further five hours (Gerlier and Thomasset 1986), prior to removal of the media under vacuum, and thoroughly washing the wells (3x) with HBBS. The degree of formazan formation was then determined on a variable wavelength VersaMax spectrophotometer at 570nm (corrected to a 1cm path length) after the addition of 50µl of DMSO. Cell survival was evaluated as a percentage of viable cells in treated versus untreated wells on the same plate (% Cell

Viability). All assays were repeated nine times. Results are presented as mean ± standard deviation.

The amount of reactive oxygen species (ROS) present was determined by the addition of dichlorofluorescin diacetate (DCFH-DA) dye to the culture media for 30 minutes, prior to removal of the media under vaccum, and thoroughly washing the wells (3x) with HBBS. The degree of DCF formation was determined at 504 nm (Gomez-Lechon *et al.*, 2003; Somogyi *et al.,* 2007) and converted to %ROS production (ROS of treated well in comparison to the control well as a percentage). All assays were repeated nine times. Results are presented as mean ± standard deviation.

3.3.5 Toxicity assay with diclofenac and meloxicam: Cell survival and ROS production in the presence of Urica Acid

The same culture method as above was used, however 0.005mg/ml (5µl) was added into each well. The concentration was calculated to represents homeostatic uric acid concentrations for the birds. The assumption of the addition of uric acid, was that toxicity may be related to digestion of a high protein diet and co-dependant on uric acid being present. All assays were repeated nine times. Results are presented as mean ± standard deviation.

3.3.6.Toxicity assay with diclofenac metabolites: Cell survival and ROS production

3.3.6.1 Generation of metabolites (Pre-incubated drug)

The hepatocytes, 2000 µℓ (10^5 cells/mℓ) were seeded into 24-well plates (Lu *et al.,* 2004) and the assays were allowed 48 hours to establish themselves. The cultures were subsequently innoculated with diclofenac for 0.5, 1, 1.5 and 2 hours at 37°C on a shaker platform. After the specified incubation, the reaction was stopped by adding 1 ml of acetonitrile into the well, which already contained adherent hepatocytes in 2 ml of DMEM media. The cell contents were subsequently dried under a stream of nitrogen to produce an extract of 1ml in water. The extract produced was assumed to be rich in metabolites, as result of the in vivo half-life of diclofenac in the crow. The established RTE cell and hepatocytes were subsequently exposed to 10 µℓ of the metabolite extract in exactly the same manner as used for the assay

using pure diclofenac. Due to financial constraints, quantification of the change in diclofenac concentration was not possible. However, the protocol did, allow for diclofenac quantification if overt toxicity was constantly present in the metabolite fraction.

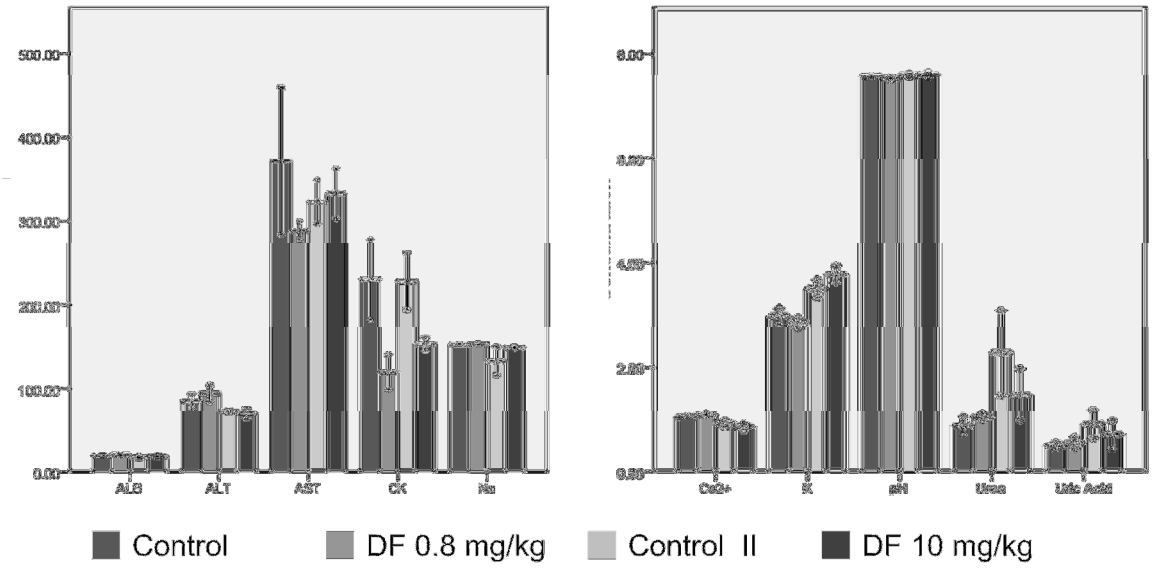
CHAPTER

4

4.1. RESULTS

4.1.1. In vivo Toxicity study

The birds remained healthy throughout the treatment period despite being dosed at 0.8 mg/kg (the vulture toxic dose) and 10mg/kg (the oral chicken LD_{50} dose). Following scheduled termination, the birds were euthanized, samples collected and submitted for clinical and gross pathological evaluations. No lesions were evident on gross pathology and all clinical pathology parameters monitored during the study remained within normal limits (Fig 4.1).



Albumin (Alb)(g/ℓ) alanine aminotransferase (ALT) Aspartate aminotransminase (AST) creatine kinase (CK), sodium (Na^+)(mmol/ℓ), calcium (Ca^{2+})(mmol/)ℓ, potassium (K^+)(mmol/ℓ), Uric acid (UA) (umol/ℓ), Urea(U/)ℓ),

Figure:4.1: Clinical pathology parameters evaluated in the control and diclofenac treated birds, at doses of 0.8mg/kg and 10 mg/kg (n=6). Results are presented as mean ± SEM. The control groups were dosed with water.

Table 4.1 : Estimated pharmacokinetics parameters for the crow following the administration of diclofenac at 10 mg/kg per os.

Parameter	Unit	Mean
Lz	1/h	
Thalf (T1/2)	h	2.33
AUC	mg/ml*h	0.54
Clearance	L/h*kg	17.36
Vd	L/kg	58.35

AUC: Area under the plasma concentration versus time profile; Thalf: Half-life of elimination; Vd: Volume of Distribution; Lz: Elimination rate constant.

No diclofenac residue was detectable in any of the tissues matrices analysed or within the plasma of the 0.8 mg/kg treated crows. The drug was however detectable in the plasma of the crows dosed at 10 mg/kg at each of the 4 and 12 hours periods, with a mean concentration of 0.1 and 0.01 µg/ mℓ respectively. While insufficient plasmas time points were attained for a full pharmacokinetic analysis, partial curve fitting revealed a plasma half-life of approximately 2.4 hours ((Table 4.1). Diclofenac was also characterised by a small area under the curve of 0.54 µg/mℓ *h, extensive volume of distribution of 58.35 L/kg and rapid clearance of 17.36 L/h*kg. Nonetheless these parameters are only used as a gross estimate for the discussion and are not actual pharmacokinetics parameters for this species.

4.1.2. IN VITRO CULTURE RESULTS

The cell culture results following exposure of renal tubular epithelial cells and hepatocytes with either pure drug or a drug metabolite rich fraction are presented in Tables 4.2 to 4.4 and Figures 4.2 to 4.9 below. The information is presented as mean and standard deviation for each concentration tested. Pharmacodynamic analysis of the different data points were not undertaken as the results were not linear over time or concentration. As a result the EC_{50} and EC_{max} effect of the drugs tested could not be established. Statistical analysis was not deemed necessary as visual analysis and the overlapping SD clearly indicated that no significance differences were evident. As per normal statistical analysis, means need to be around 2 X SD different for the test to pick up a significant difference.

Table 4.2: Mean and SD of the % cell viability (MTT) obtained following incubation of renal tubular epithelial cells with either pure drug or a drug metabolite rich fraction

Treatments	Drug form	Concentration (µM)							
		80		40		20		10	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
DF	1	81	22	91	15	87	22	93	15
	2	96	6	96	13	96	9	96	7
DF+UA	1	88	18	90	13	79	22	89	14
	2	81	28	83	26	82	28	84	28
MLX	1	77	36	80	34	71	36	77	36
	2	85	29	87	28	85	29	86	28
MLX+UA	1	69	37	69	37	64	36	68	39
	2	76	33	80	29	79	31	82	31

DF- diclofenac; DF+UA – Diclofenac incubated in the presence of uric acid; MLX- Meloxicam; MLX + UA – Meloxicam incubated in the presence of uric acid. 1- Incubated as pure drug obtained from Sigma South Africa; 2- Incubated as metabolite rich fraction

Table 4.3: Mean and SD of the % cell viability (MTT) obtained following incubation of hepatocytes with either pure drug or a drug metabolite rich fraction

Treatments	Drug form	Concentration (µM)							
		80		40		20		10	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
DF	1	82	26	78	27	79	27	75	30
	2	84	26	82	27	84	25	84	25
DF+UA	1	94	15	90	17	89	19	94	17
	2	87	27	90	22	85	25	87	20
MLX	1	62	39	55	39	54	38	55	39
	2	70	35	72	35	77	32	74	35
MLX+UA	1	50	42	49	42	50	43	45	42
	2	66	38	71	38	70	39	70	37

DF- diclofenac; DF+UA – Diclofenac incubated in the presence of uric acid; MLX- Meloxicam; MLX + UA – Meloxicam incubated in the presence of uric acid. 1- Incubated as pure drug obtained from Sigma South Africa; 2- Incubated as metabolite rich fraction

Table 4.4: Mean and SD of the % ROS production obtained following incubation of renal tubular epithelial cells with either pure drug or a drug metabolite rich fraction

Treatments	Drug form	Concentration (µM)							
		80		40		20		10	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
DF	1	57	43	60	40	58	43	63	39
	2	57	45	57	44	57	45	57	45
DF+UA	1	59	39	61	39	59	39	62	40
	2	74	36	77	34	64	41	69	38
MLX	1	68	38	70	37	68	39	71	39
	2	80	35	70	39	71	40	72	39
MLX+UA	1	62	41	64	40	66	40	66	41
	2	77	35	73	37	71	39	78	35

DF- diclofenac; DF+UA – Diclofenac incubated in the presence of uric acid; MLX- Meloxicam; MLX + UA – Meloxicam incubated in the presence of uric acid. 1- Incubated as pure drug obtained from Sigma South Africa; 2- Incubated as metabolite rich fraction

Table 4. 5: Mean and SD of the % ROS production obtained following incubation of hepatocytes with either pure drug or a drug metabolite rich fraction

Treatments	Drug form	Concentration (µM)							
		80		40		20		10	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
DF	1	61	41	62	42	63	42	60	42
	2	58	44	59	45	70	42	58	43
DF+UA	1	65	41	65	40	65	38	67	39
	2	75	35	77	33	82	30	78	30
MLX	1	51	40	44	39	45	40	44	39
	2	60	41	60	40	67	38	61	40
MLX+UA	1	50	42	49	43	50	43	49	43
	2	70	38	71	38	69	38	71	38

DF- diclofenac; DF+UA – Diclofenac incubated in the presence of uric acid; MLX- Meloxicam; MLX + UA – Meloxicam incubated in the presence of uric acid. 1- Incubated as pure drug obtained from Sigma South Africa; 2- Incubated as metabolite rich fraction

4.1.2.1. Description of the results for the MTT assay

a) Diclofenac in the presence and absence of uric acid

Following incubation with diclofenac, both the RTE and hepatocytes showed minimal signs of toxicity (± 80% cell viability) in the absence or presence of uric acid (Fig 4.2). When differences between the groups were compared, the degree of toxicity in the presence of uric acid appeared to be lower albeit in a non-significantly different manner. No time related trends were evident, with the 24hour time point appearing to induce the greatest degree of toxicity. This could be seen in all the wells that were exposed to the different concentration of diclofenac in the absence of uric acid (Figure. 4.2, A to C and Table 4.2). When the toxicity between the two cell-types is compared, the hepatocytes appeared to be as insensitive as the RTE cells to diclofenac.

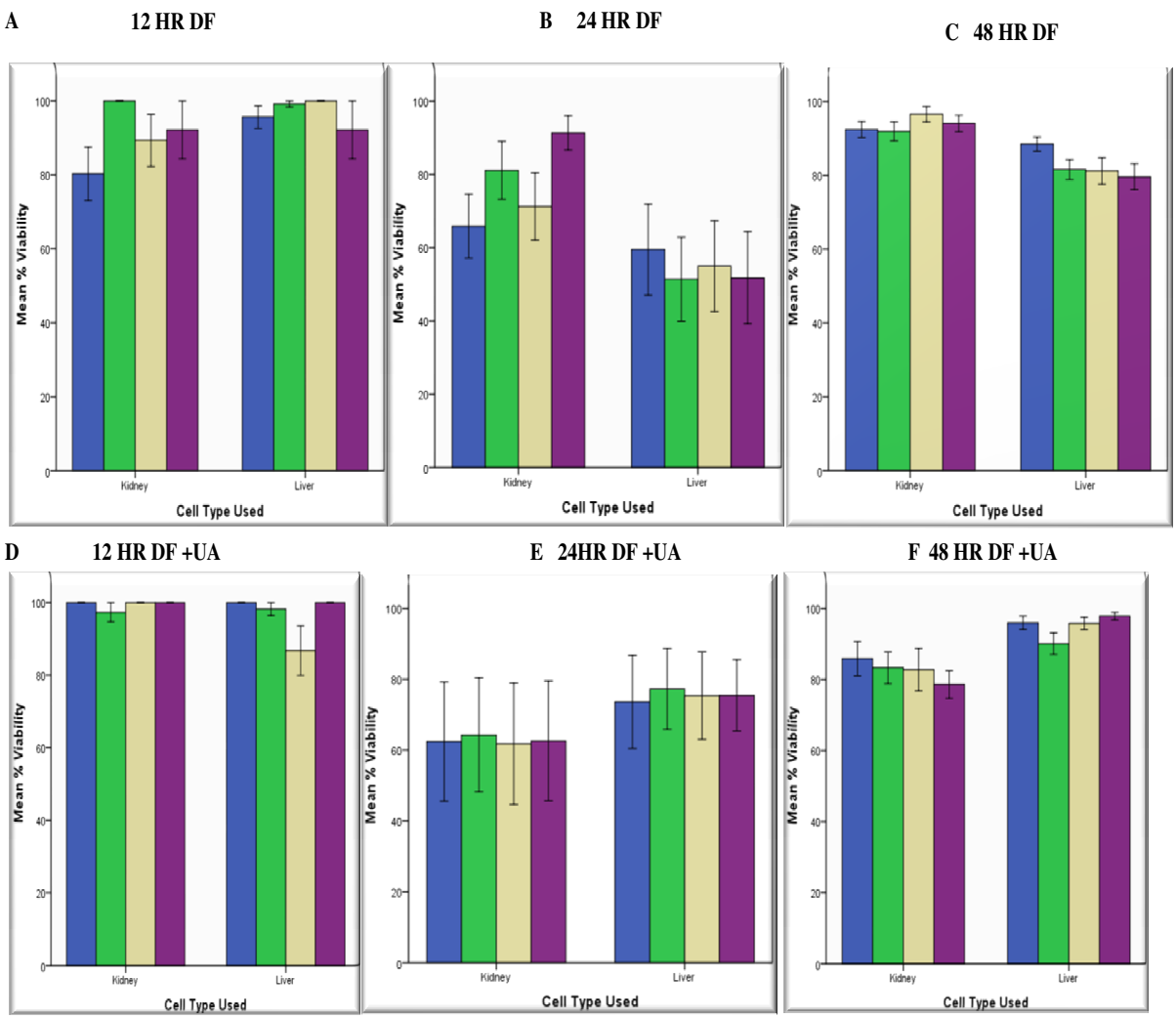


Figure 4.2: Cell viability following incubation with diclofenac for renal tubular epithelial (RTE) cells or hepatocytes. Results are presented as mean ±1 SE and from left to right are the 12, 24 and 48 hours periods of incubation. Culture was incubated in the absence (A-C) or presence (D-F) of uric acid. Four concentrations were tested 10 ,20, 40, and 80µM from left to right.

b) ***Meloxicam in the presence and absence of uric acid***

In contrast to diclofenac, meloxicam appeared to be moderately toxic (40-80% cell viability) to the RTE cells at all time-points.(Figure 4.3 and Table 4.2). For the hepatocytes, marked toxicity (<40% cell viability) was evident in only the 12 hours sample, in both the presence or absence of uric acid (Figure 4.3A and D), while the 24 to 48hrs time points demonstrated only mild toxicity. With the exception of the 12 hour results the hepatocyte toxicity results also demonstrated a large standard error. The addition of uric acid to the media appeared to have no additional influence on toxicity.

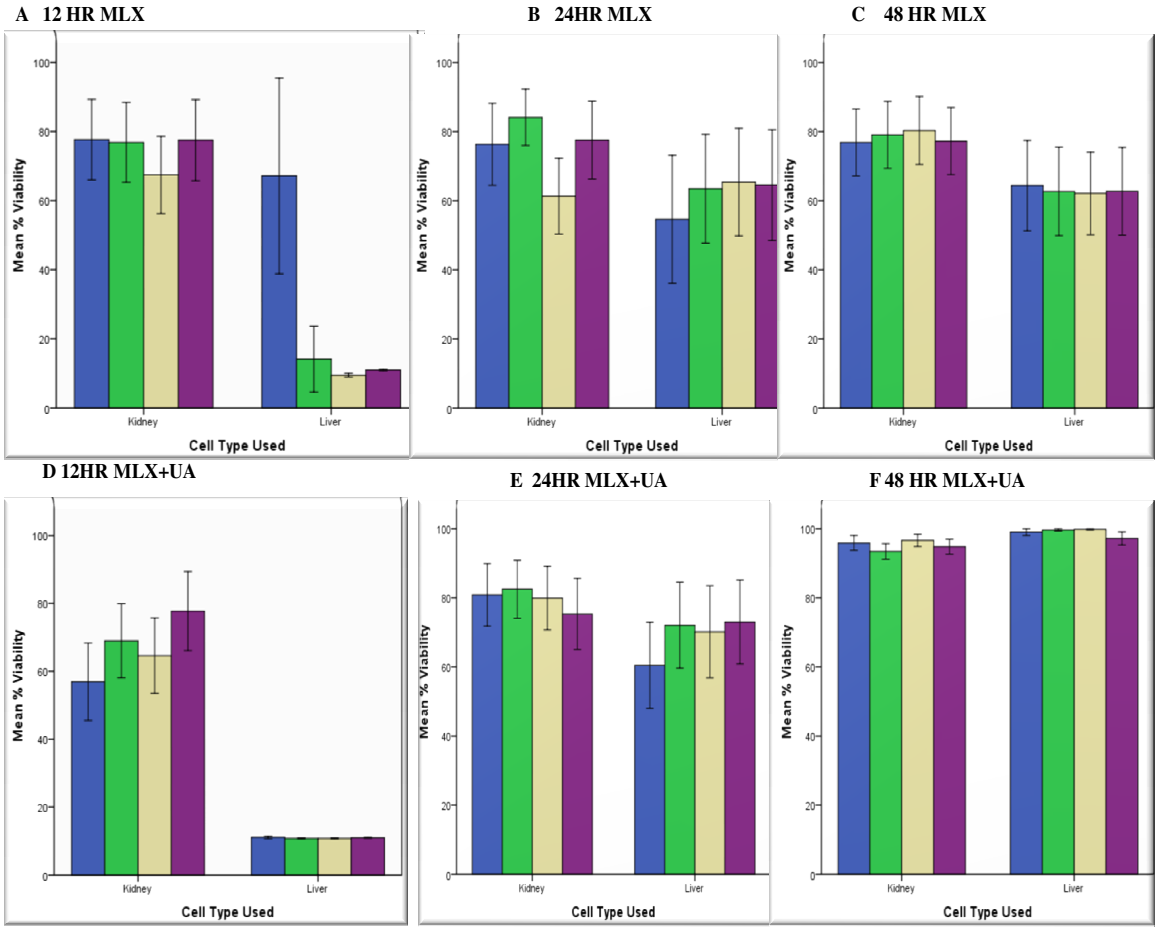


Figure 4.3: Cell viability following incubation with meloxicam for renal tubular epithelial (RTE) cells or hepatocytes. Results are presented as mean ± 1 SE and from left to right are the 12, 24 and 48 hours periods of incubation. Cultures were incubated in the absence or presence (A-C) of uric acid (D-F). Four concentrations were tested 2.5, 5, 10, and 20 µM from left to right.

c) Pre-incubated *Diclofenac in the presence and absence of uric acid*

The diclofenac metabolite fraction showed minimal toxicity in the first 12 hours (Fig 4.4). Moderate toxicity was evident hereafter, with 24 hour sample showing the largest degree of toxicity. No difference was evident in the presence or absence of uric acid.

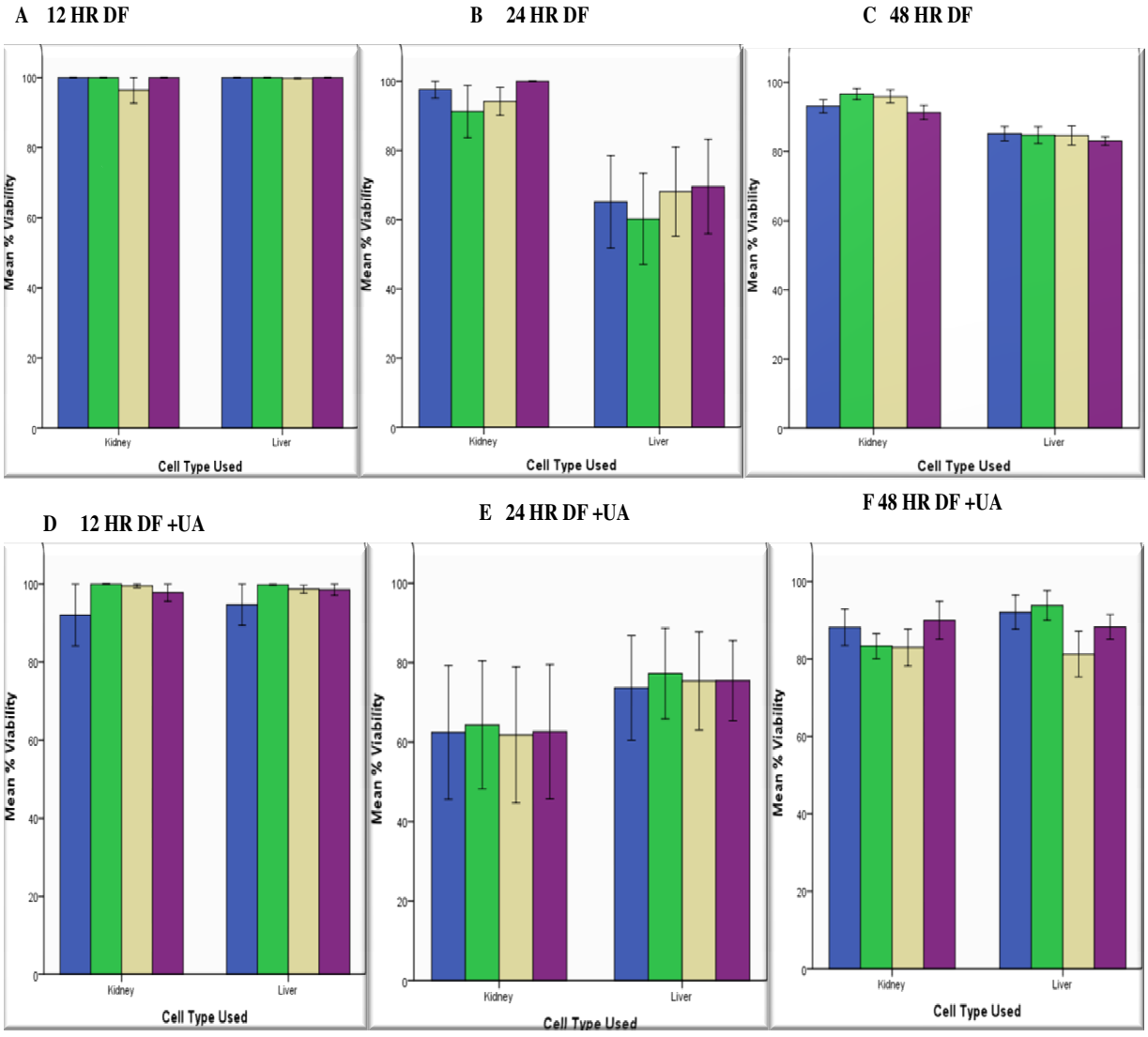


Figure 4.4: Cell viability following incubation with diclofenac metabolite fraction for renal tubular epithelial (RTE) cells or hepatocytes. Results are presented as mean ± 1 SE and from left to right are the 12, 24 and 48 hours periods of incubation. Cultures were incubated in the absence or presence (A-C) of uric acid (D-F). Four concentrations were tested 10, 20, 40, and 80µM from left to right.

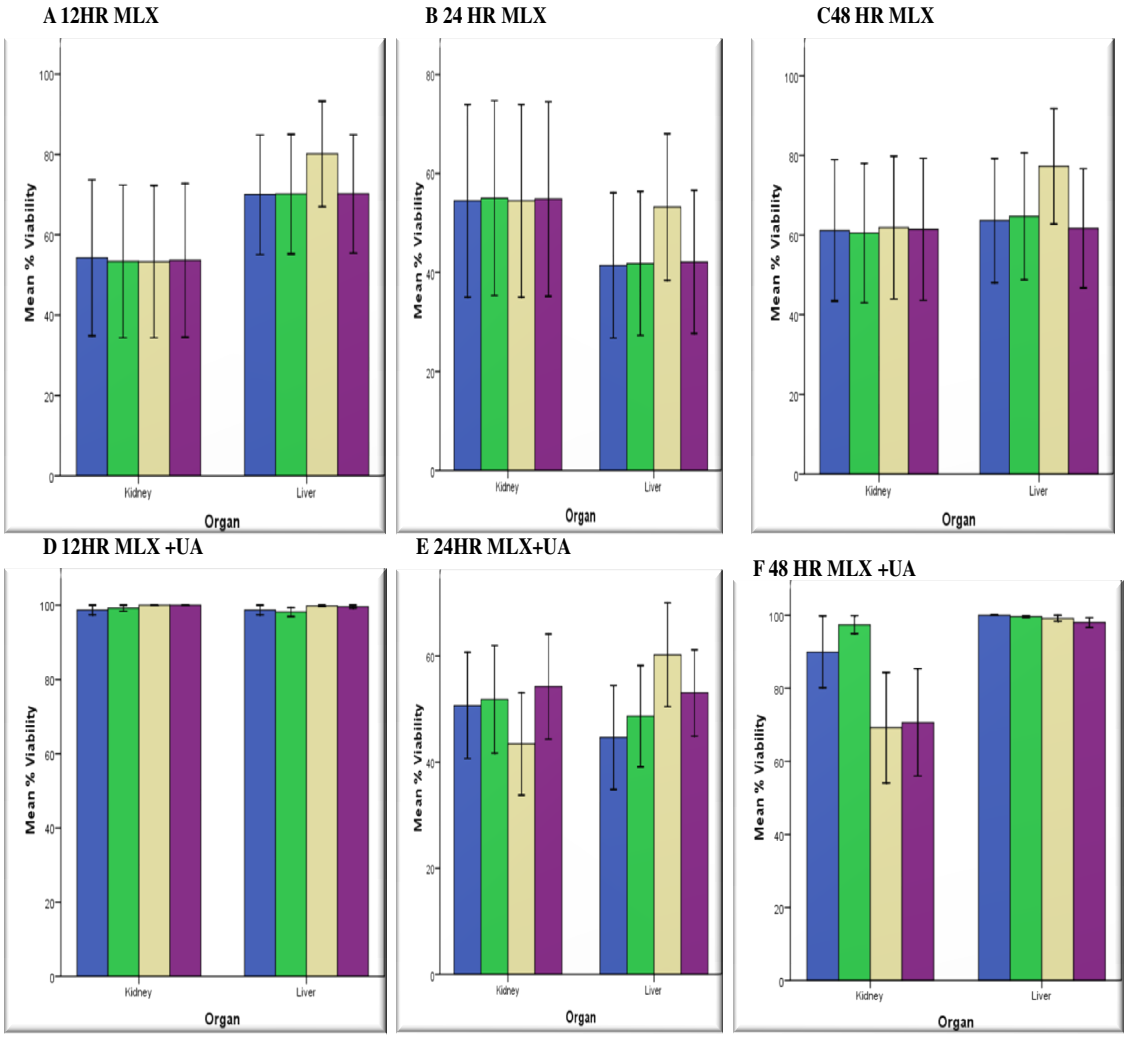


Figure 4.5: Cell viability following incubation with meloxicam metabolite fraction for renal tubular epithelial (RTE) cells or hepatocytes. Results are presented as mean ± 1 SE and from left to right are the 12, 24 and 48 hours periods of incubation. Cultures were incubated in the absence or presence (A-C) of uric acid (D-F). Four concentrations were tested 2.5,5, 10 and 20µM from left to right

4.1.2.2. Description of the results for the ROS assay

Neither diclofenac, meloxicam or their metabolite rich reactions induced an increase in ROS production in the hepatocyte and RTE cell culture (Figure 4.6 to 4.9). For all wells, the ROS concentrations decreased in comparison with that of the control wells (results presented as a percentage of the control well's concentration).

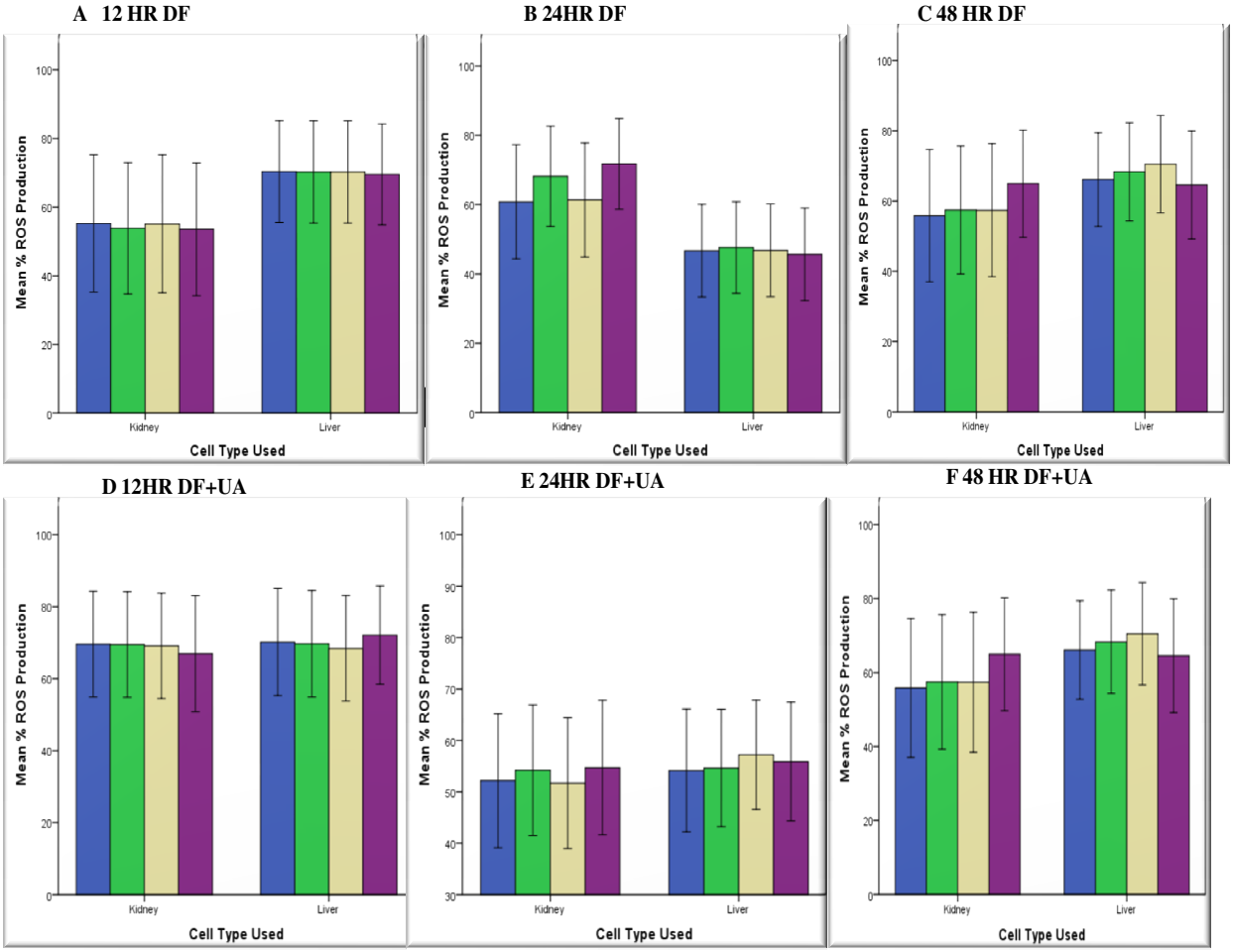


Figure 4.6: % Reactive oxygen species (ROS) formation following incubation with DF for renal tubular epithelial (RTE) cells or hepatocytes. Results are presented as mean ± 1SE and from left to right are the 12, 24 and 48 hours periods of incubation. Cultures were incubated in the absence (A-C) or presence of uric acid A- C (D- F). Concentration of DF are 10 ,20, 40, and 80 µM from left to right.

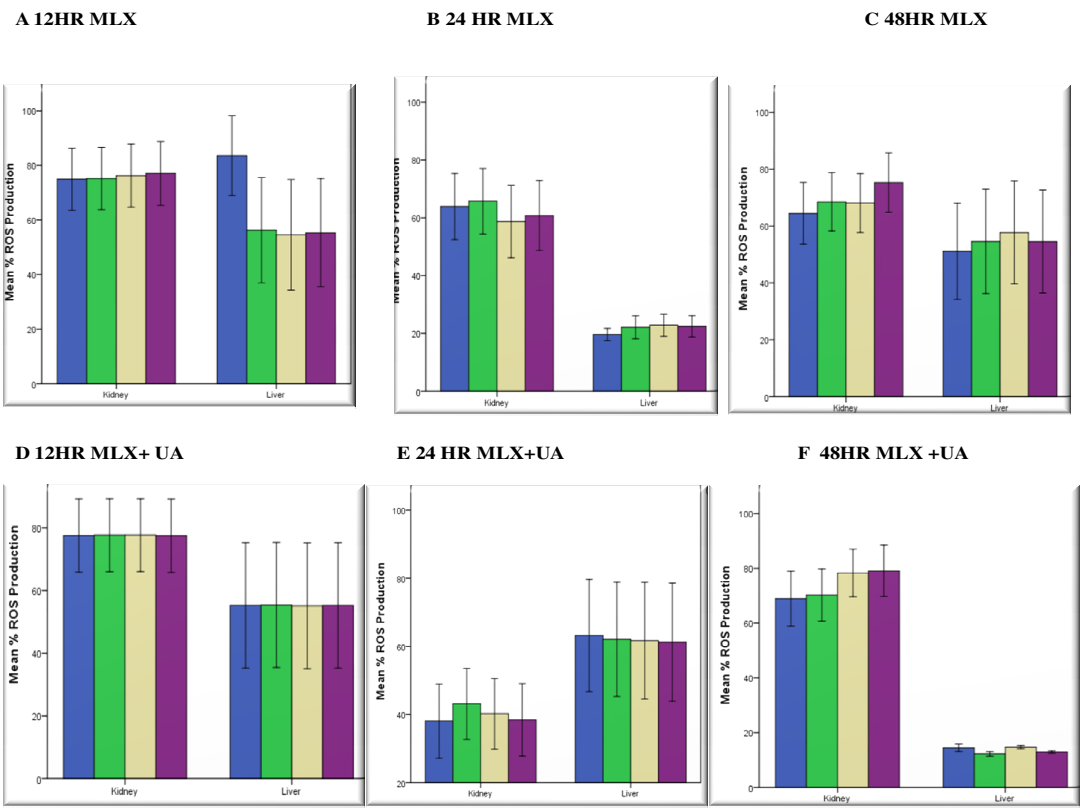


Figure. 4.7: % ROS formation following incubation with meloxicam. Results are presented as mean ± 1 SE and from left to right are the 12, 24 and 48 hours periods of incubation. Cultures were incubated in the absence (A-C) or presence of uric acid (D- F). Concentration of MLX are2.5, 5, 10, and 20 µM from left to right.

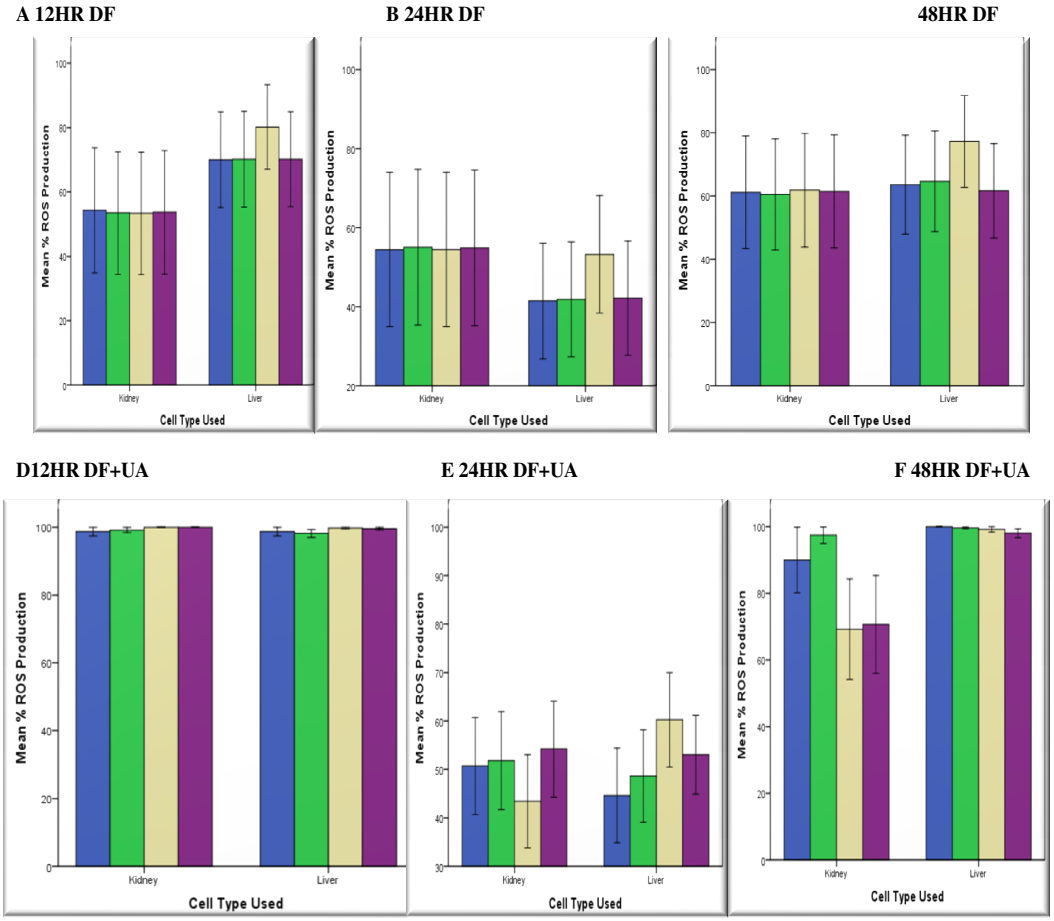


Figure 4.8: % Reactive oxygen species (ROS) formation following incubation with DF metabolite for renal tubular epithelial (RTE) cells or hepatocytes. Results are presented as mean ± 1SE and from left to right are the 12, 24 and 48 hours periods of incubation. Cultures were incubated in the absence (A-C) or presence of uric acid (D-F) from the 0.5, 1, 1.5 and 2h wellsfrom left to right.

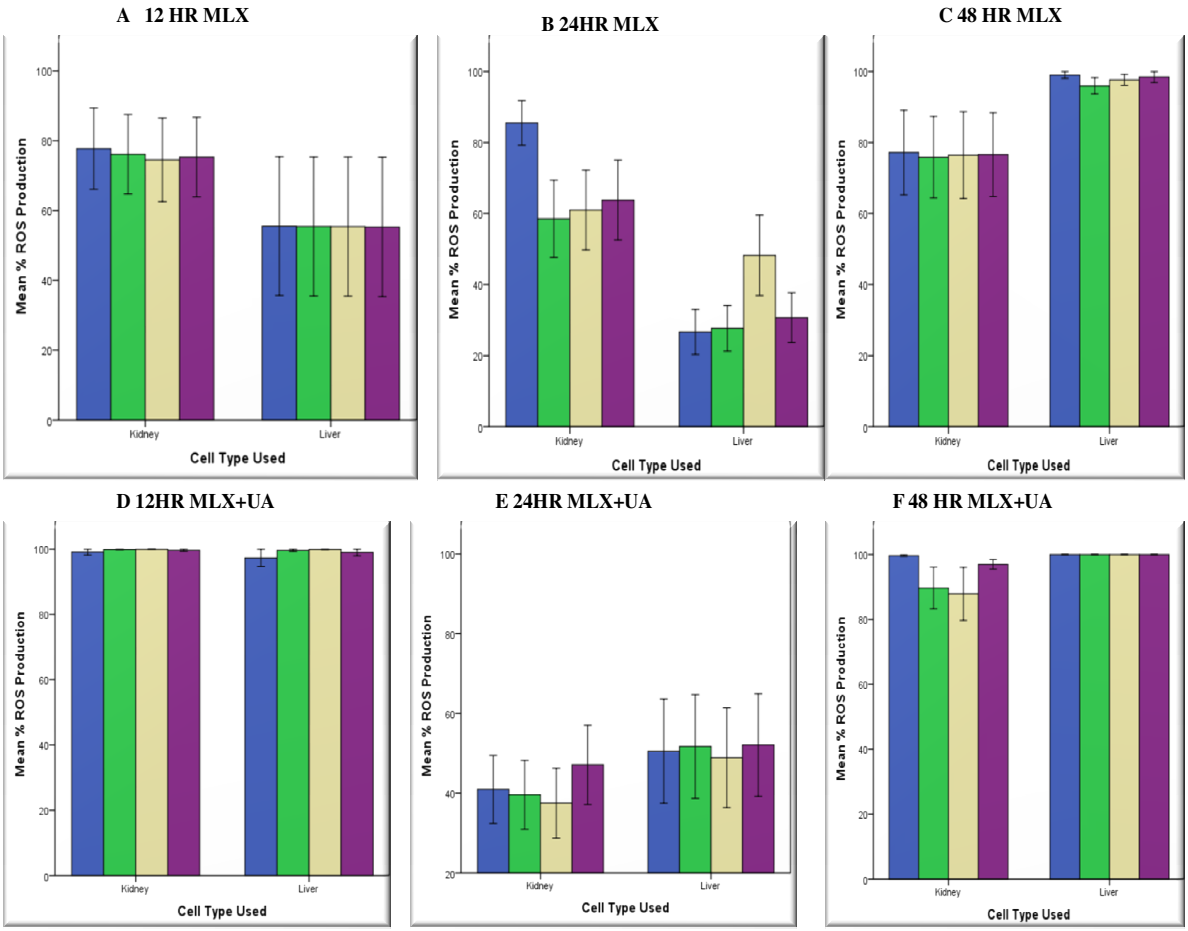


Figure 4.9: Reactive oxygen species formation measured within renal tubular epithelial (RTE) cells or hepatocytes following incubation MLX metabolite fraction Results are presented as mean ±1 SE and from left to right are the 12, 24 and 48 hours periods of incubation. Culture were incubated in the absence or presence of uric acid A- C (absence) D- F (Presence of uric acid) from the 0.5, 1, 1.5 and 2h wells from left to right.

CHAPTER

5

5.1Discussion

The crows were surprisingly tolerable to the effects of diclofenac in comparison to the Gyps vulture and the chicken. While both the chicken (10 mg/kg) and vulture (0.8 mg/kg) showed identical signs of toxicity albeit at the different doses listed viz. severe renal toxicity and visceral gout this was completely absent in the crow at both concentrations identified as being toxic in the latter species. While the reason for the difference in toxicity is not currently known, differences in species metabolism or alternatively differences in the renal cell susceptibility to toxicity may be the reason for the non-susceptibility seen. To obtain a better understanding of differences in interspecies metabolism, the sparse data obtained from the crows were used in calculating crude pharmacokinetic parameters (which are more likely to be an overestimate). The results cannot be considered accurate as the LLQ concentration was used for the last time point which is inherently inaccurate as this concentration could have been achieved anywhere between the 4 and 12 hour sampling points.

For the crow, diclofenac was characterised by a rapid half-life of elimination which was much shorter than that observed in turkey vulture and in *Gyps* vulture species (Swan et al., 2006b, Rattner et al., 2008, Naidoo *et al*., 2009). It was however twice that reported for the domestic chicken at the same dose of 0.8 mg/kg (as evaluated in a full pharmacokinetic study), for which no signs of toxicity were evident. The crow profile was also characterised by very low plasma DF concentrations, and in turn a low level of exposure, thereby indicating that overall exposure in the systemic circulation was low (reflected by the large Vd). Considering that Vd for the NSAIDs in general has been small in other bird species, it is believed that the large Vdsupports rapid metabolism (Naidoo *et al*, 2009). Based on the presystemic elimination evident for ketoprofen in both vulture and people, pre-systemic elimination may also being concurrently occurring in the Pied crows. As a result it may be interesting to establish the intravenous toxicity

and toxicokinetics of diclofenac in the crow, as it may well be that in the absence of presystemic elimination, diclofenac may well prove to be toxic(Naidoo et al, 2009).

To further illustrate the importance of metabolism in toxicity, the pharmacokinetic parameters obtained were compared to that for other avian species(Table5.1) (Swan et al.,2006b, Naidoo et al., 2007, Rattner et al., 2008, Naidoo et al., 2009). In all cases, where plasma concentration data was available, birds tended to die when half-lives were longer than 12 hrs. However, when the half-life was <7 hrs, animals survived. This observation was marked in the chicken, where a low dose resulted in no mortality and showed a half-life of 1 hr, whilst at a 5 mg/kg dose, the one bird treated died, and displayed a half-life profile of 14 hrs. This follows a similar finding reported in a pharmacokinetic study of ketoprofen in Cape Griffon vultures (*Gyps coprotheres*) where toxicity occurred at higher doses, when half-lives also indicated zero order metabolism (Naidoo et al., 2010a).By zero order pharmacokinetics, it is implied that the liver (or related organ of metabolism), fails to adequately metabolise the drug in timely manner. As a result of this limitation, the drug accumulates within the body until the levels become high enough to cause toxicity.

Table 5.1.: Estimated pharmacokinetic parameters for various avian species dosed with diclofenac.

Species	Common name	n	Route	Dose (mg/kg)	Status	t½ (h)	Cl (L/h*kg)	AUC (µg/ml*h)	Ref
Gallus domesticus	Chicken	6	oral	0.8	Alive	0.89	0.189	4.33	Naidoo et al, 2007
Corvus albus	Pied crow	2	oral	10	Alive	2.33	17.36		
Cathartes aura	Turkey vulture	2	oral	25	Alive	6.29	0.26	141.15	Rattner et al, 2008
Cathartes aura	Turkey vulture	2	oral	8	Alive	6.43	0.79	13.86	
Gyps coprotheres	Cape griffon vulture	2	oral	0.8	Died	12.24	0.01	80.28	Naidoo et al, 2009
Gallus domesticus	Chicken	1	im	5	Died	14.34	0.65	6.30	Naidoo et al, 2007
Gyps africanus	African white backed	2	oral	0.8	Died	16.78	0.02	61.95	

Values for all species, except *G. domesticus* at 0.8 mg/kg oral route, were estimated using equations described for non-compartmental modelling.

im = intramuscular, t½ = half-life of elimination, AUC = area under the plasma concentration versus time curve, Cl = Plasma Clearance.

To better explain the absence of toxicity at the tested dose and route of exposure, a second experiment was designed to ascertain if toxicity was related to *ex vivo* cellular insensitivity to diclofenac or whether it was just metabolism was protective, as seen in the vulture for meloxicam viz meloxicam was toxic in cell cultures after long-term exposure which was substantially greater than less than the mean residence time of the drug in vivo (Naidoo *et al*, 2007; Naidoo *et al*, 2006).For this study, evidence of toxicity was based on direct cell toxicity (MTT assay) and ROS production (DCFH-DA method) as previous literature has shown that diclofenac's toxicity in mammals resulted from the bioactivation of the parent compound to a reactive metabolite, which may be capable of either modifying biological material covalently or participating in redox-cycling reactions (Bolton et al, 2000). Within the cell the mitochondria appears to be the prime target, as this organelle is very susceptible to the effects of ROS. The MTT assay evaluates cell toxicity based on the ability of a viable mitochondria to convert MTT to formazin.

Another interesting feature of diclofenac's toxicity is that cell-level metabolism plays an important role in toxicity. This effect was elegantly demonstrated by Bort et al., 1998, when the toxic effects of diclofenac was examined on human and rat primary hepatocytes and two hepatic cell lines (HepG2, FaO). The study was able to demonstrate the importance that metabolism played in toxicity as diclofenac was more cytotoxic to drug metabolizing cells. The estimated IC_{50} values for primary rat and human hepatocytes (concentration causing 50% cell death) were closely related, an IC_{50} of : 392 ± 34 µM (*n* 5=16) and 331 ±7 µM (*n* = 6), respectively. Non hepatic cells (MDCK cell line) were tested and found to be less sensitive to diclofenac at an IC_{50} of 763 ±61 µM, (*n* 4) and FaO IC_{50} of 682 ±64 µM, (*n* 4).The curves demonstrating toxicity shifted towards higher concentration of the drug.

One modification made to this study was the inclusion of uric acid into some of the cell cultures. This inclusion was per chance toxicity was related to ROS, as uric acid has been shown to be an important intracellular anti-oxidant. In addition, toxicity in the vulture has always been associated with an increase in plasma uric acid. This has led to the speculation by (Naidoo et al, 2007) that diclofenac may be indirectly toxic by interfering with the intracellular regulation of uric acid. For the latter toxicity would result either from the uric

acid killing the cell or more likely that the cell dies from apoptosis as it is being denied an important anti-oxidant system.

For this study primary renal and hepatocyte cultures were exposed to four concentrations of diclofenac or meloxicam for 12, 24 or 48 hours. The long period of exposure (48 hours) was used to simulate an extremely long mean residence time *in vivo*, which corresponded to the maximum incubation period observed in other studies (Ng *et al*, 2008, Naidoo *et al*, 2009). In addition, the concentrations used were significantly larger than those actual concentrations measured in the crow at 4 hours in the pharmacokinetic profile.For the RTE cells and hepatocytes, meloxicam and diclofenac were similarly non-toxic for the majority of the time points examined in both the presence and absence of uric acid. A major exception was, however, present for hepatocytes especially for the 12 hour sampling both in the presence and absence of uric acid. While the degree of toxicity at 12 h was similar to that seen for the chicken RTE cell cultures, the absence of significant toxicity at 24 and 48hrs is difficult to explain. One possible reason initially considered was an unexpected death of cells on the particular plate, which is probably non-important, as the assay was repeated in triplicate at one week intervals. The only other reason, albeit unlikely, was that the hepatocytes have become undifferentiated and had started dividing by 24 hours. In studies with rat hepatocytes, it was shown that non-differentiated hepatocytes were less sensitive to diclofenac than primary cultures of hepatocytes (Bort et al., 1998) which resulted from the loss of enzymes within the hepatocytes. The primary tissue mass of hepatocytes was filtered so as to minimize fibroblast contamination, although no molecular characterization was done to check whether any fibroblast contamination was present, the duration of incubation was only 48 hours. Hepatocytes were continuously checked under microscope. Only red blood cells and hepatocytes were seen.

In order to determine if bio-activation played an important role in toxicity, cell cultures with a high concentration of hepatocytes were exposed to diclofenac and meloxicam for various periods of time prior to harvest, to generate a metabolite rich fraction. The culture fluid from these wells were subsequently extracted and used for toxicity assays. No difference in toxicity was evident in between the parent drug and the drug metabolite fraction for both meloxicam and diclofenac. While it may be argued that the hepatocytes were unable to metabolize the diclofenac and meloxicam added therein, the more likely scenario was the

failure of the cells to induce any metabolism in the period of testing. Unfortunately due to financial constraints, the change in concentration of diclofenac and meloxicam in the wells could not be quantified. Further evidence in support of a failed culture assay, can be seen with the presence of toxicity of meloxicam at 12 h for the hepatocyte cultures (Fig 4.3A) to the same degree as seen for the pure drug (Fig 4.4A). This suggests that the concentration of meloxicam in the wells were the same i.e. the culture failed to metabolise the drug.

As mentioned above, an important factor in toxicity of diclofenac and meloxicam is the increase in the production of ROS at the level of the mitochondria. To ascertain if the absence of toxicity was linked to a lack of ROS generation within the RTE and hepatocytes, intracellular ROS concentrations were quantified. As predicted from the absence of significant toxicity, ROS production within the cell cultures were minimally changed in comparison to the controls. It would therefore appear that the toxicity of diclofenac and meloxicam probably did not involve ROS production. The failure of an increase in ROS at 12 hrs corresponding to the period of high hepatocyte death was another unexpected finding. This therefore leads to the speculation that the hepatocyte death at the 12 hr point was not due to diclofenac and another reason was responsible for the cell death seen.

5.2. Conclusion

Our study demonstrates that the crow is not sensitive to effects of diclofenac. When all the culture results are evaluated as a unit, an interesting finding is present for diclofenac as the drug demonstrated no toxicity as either pure drug or as the metabolite rich fraction in combination with a lack of ROS production. Considering that the hepatocytes were at times exposed to diclofenac for 48 hours, one would have expected some bioactivation and toxicity. Therefore the lack of toxicity and short half-life *in vivo*, in combination with absence of toxicity *ex vivo* and lack of ROS activation suggests that the crow makes use of an alternate metabolic pathway to that used by vulture and chicken. The hypothesis,that diclofenac is characterised by a prolonged elimination half – life and a low LD50 in the pied crow is therefore rejected.

5.3*Future trends*

Since it has been established that biotransformation enzymes play a role in the toxicity of diclofenac and that crows are not sufficiently related to vultures for future studies, it may be of interest to ascertain why and how crows are physiologically protected from diclofenac toxicity. Perhaps an evaluation of the similarities and differences between the crow and vulture, may allow for a better understanding of the mechanism of toxicity of diclofenac in the vulture. The information may also prove to be of value in the design of potential *in vitro* screening assays. The next step would be to establish the complete intravenous pharmacokinetics of diclofenac in the crow.

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APPENDIX 1: MANUSCRIPT ACCEPTED BY WILDLIFE JOURNAL OF WILDLIFE DISEASES

NAIDOO V, MOMPATI KF, DUNCAN N, AND TAGGART MA. 2001. The pied crow (*Corvus Albus*) is insensitive to diclofenac at concentrations present in carrion. Journal of Wildlife Diseases 47: 936–944

Journal of Wildlife Diseases, 47(4), 2011, pp. 936–944

THE PIED CROW (*CORVUS ALBUS*) IS INSENSITIVE TO DICLOFENAC AT CONCENTRATIONS PRESENT IN CARRION

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ABSTRACT: Diclofenac, a nonsteroidal anti-inflammatory drug (NSAID), kills vultures (*Gyps* spp.) that consume tainted carcasses. As a result, vulture populations in India, Nepal, and Pakistan have been devastated. Studies on meloxicam and ketoprofen demonstrated that the toxicity of the NSAIDs is unpredictable, thereby necessitating individual testing of all available NSAIDs. Because it is no longer practical to use vultures for toxicity testing, we evaluated the Pied Crow (*Corvus albus*) as a model. Pied Crows ($n=6$) were exposed to a dose of 0.8 and 10 mg/kg of diclofenac, with no signs of toxicity, and a rapid half-life of elimination. Using primary renal cell and hepatocyte cultures, a high tolerance was demonstrated at the cellular level. Meta-analysis of pharmacokinetic data for the Domestic Chicken (*Gallus gallus*) and the African White-backed (*Gyps africanus*), Cape Griffon (*Gyps coprotheres*), and Turkey Vultures (*Cathartes aura*) showed a trend toward toxicity when the half-life of elimination increased. We conclude that the crow is not susceptible to diclofenac and, more important, that toxicity in the *Gyps* species is probably related to zero-order metabolism.

Key words: Avian conservation, crow, diclofenac, vulture, vulture restaurants.

INTRODUCTION

The *Gyps* spp. vulture populations on the Indian subcontinent have been devastated during the past 10–15 yr following their exposure to residues of diclofenac in the carrion they eat (Oaks et al., 2004; Prakash et al., 2007). Although this exposure was accidental, the effect was unprecedented and resulted in global species declines nearing 99.9% (Cuthbert et al., 2007). Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) often used in cattle on the Indian subcontinent because of its anti-inflammatory action, analgesic properties, low cost, and widespread availability (Swan et al., 2006b). Diclofenac became the regional NSAID of choice, and large numbers of animals received the drug before death; because the Indian cow is sacred in Hindu religion, it often dies naturally rather than being slaughtered for meat at a young age. In addition, open disposal of livestock is practiced and scavengers, including vultures have year-round widespread access to carrion now known to contain high levels of diclofenac and other NSAID residues (Taggart et al., 2009).

Although the cause of the *Gyps* vulture population decline has been conclusively linked to the widespread use and toxicity of diclofenac, the potential toxicity of other NSAIDs to scavengers is in question, especially because studies also show ketoprofen to be toxic (Naidoo et al., 2010a, b). Although it is important to determine the safety of these drugs in *Gyps* spp. vultures to protect the remaining populations on the Indian subcontinent, it is equally important to recognize risks to worldwide populations of various species of vultures and other avian scavengers. The latter group of birds is probably equally at risk of accidental toxicity as many countries (Namibia, Spain, India, Italy, Israel) have introduced "vulture restaurants," where colonies are artificially fed through routine placement of carcasses (Meretsky and Mannan 1999; Bamford et al., 2007; Gilbert et al., 2007; Cortes-Avizanda et al., 2009; Gustin et al., 2009; Margalida et al., 2009). Hence, if

any other NSAIDs (e.g., carprofen, flunixin meglumine, phenylbutazone, tolfenamic acid, meclofenamic acid, and ibuprofen) were to prove as toxic as diclofenac and ketoprofen, these vulture populations might also be at risk.

The optimal way to conclusively determine whether other NSAIDs are safe is to investigate their in vivo toxicity. Although this is theoretically possible using captive vultures, there are a large number of NSAIDs available, many of which may also be administered in combinations. Because most vulture species, including *Gyps* spp., are now regionally or globally endangered (IUCN, 2010), in vivo trials on all drugs or combinations are not feasible, especially given the large sample sizes required to prove drugs to be safe (as was the case for meloxicam; Swan et al., 2006a). One alternative would be to use a bird species that is common, widely distributed, easy to handle, and not endangered. Such a species would ideally be as susceptible to all NSAIDs to be tested as are *Gyps* species. The Domestic Chicken was initially considered because it is easily available, inexpensive, and sufficiently small to handle easily. However, Naidoo et al. (2007) found that the chicken was not sufficiently susceptible to the toxic effects of diclofenac to be a suitable surrogate. The diclofenac 50% lethal dose (LD_{50}) for chickens was approximately 10 mg/kg, and the drug had a rapid half-life of elimination of <4 hr at a dose of 0.8 mg/kg. In comparison, in *Gyps* spp., the LD_{50} is estimated at 0.098–0.23 mg/kg, and the corresponding half-life is >12 hr (Naidoo et al., 2007).

Although still unproven, the different toxicity effects between chickens and *Gyps* spp. vultures may be related to different liver and kidney functions between the species. Vultures tend to be exposed to higher levels of uric acid because they consume a meat-based diet, whereas the diet of chickens is omnivorous. Therefore, vultures may be more sensitive to diclofenac because they cannot tolerate its uricosuric effects (Maier et al., 1979). Alternatively, differences may be due to the metabolic capacity of the chicken liver, in which the diclofenac half-life is much shorter, and therefore, the metabolic processing appears to be much more efficient (Naidoo et al., 2007). To eliminate one of these potential factors, we selected crows (*Corvus* spp.), which are more closely related to Old World Vultures and have similar scavenging behavior. In addition to being wild (rather than domesticated like the chicken), crows are also carrion eaters that, like vultures, generate high concentrations of uric acid daily. Our aim was to determine the sensitivity of wild-caught crows to the effects of diclofenac. We used Pied Crows (*Corvus albus*) because they are abundant and not threatened or endangered in Southern Africa.

MATERIALS AND METHODS

In vivo toxicity assay

Animal husbandry: The study was approved by the animal use and care committee of the University of Pretoria and followed ethical guidelines for the use of animals in South Africa. Twelve Pied Crows were captured using a baited cage. The birds were transported to the Poultry Reference Laboratory (Onderstepoort, South Africa) and housed in individual layer cages. Birds were identified using colored leg bands. Animals were given approximately 60 g of minced beef per day. The meat was assumed free of diclofenac and other NSAIDs because South Africa follows the CODEX ALIMENTARIUS standards in controlling drug residues in food destined for human consumption. Birds had free access to water throughout the study.

Animal treatment: Birds were dosed using a two-way crossover design. For the first phase, birds were dosed with diclofenac (Adco Diclofenac, Adcock Ingram, Johannesburg, South Africa) at 0.8 mg/kg ($n=6$) or with sterile water ($n=6$) by gavage. Following a 2-wk wash-out period, the groups were swapped (crossed), and the birds dosed again with either sterile water ($n=6$) or diclofenac at 10 mg/kg ($n=6$). To continue into the second phase of the study, a prerequisite was that there be no signs of toxicity (either clinically or

in any clinical pathology) during the first phase. No bird received more than a single dose of diclofenac during the study.

Monitoring: Animals were monitored every 2 hr for the first day, and thereafter, twice daily until 3 days postdosing for any clinical signs of toxicity. Blood samples were collected in serum tubes (Vacuette, Greiner Bio-One International AG, Kremsmuenster, Austria) for clinical chemistry or in heparin tubes (Vacuette) for diclofenac analysis before dosing and at 2, 12, 24, and 48 hr postdosing. Birds were sequentially bled so that the ethical blood volume (<1% of body weight) was not exceeded. Clinical pathology parameters evaluated were sodium (Na^+; mmol/l), potassium (K^+; mmol/l), and calcium (Ca^{2+}; mmol/l) using a RAPIDLab 34E blood gas analyzer (Chiron Diagnostics, Bayer, Johannesburg, South Africa) and uric acid (UA; U/l), alanine aminotransferase (ALT; U/l), creatine kinase (CK; U/l), and albumin (ALB; g/dl) using a Nexet Chemistry Analyzer (Alfa Wassermann, Bayer). Significant differences in changes in clinical pathology parameters were tested using a univariate analysis of variance (SPSS ver. 19, Chicago, Illinois, USA), with dose and phase used as factors.

Three days after the second phase was completed, birds were euthanized by intravenous pentobarbitone overdose and subjected to full necropsy. Liver and kidney samples were collected and frozen for diclofenac residue analysis.

Drug analysis: Tissue and plasma samples (0.5 g and 0.3 ml, respectively) were homogenized in 2 ml of acetonitrile (high performance liquid chromatography [HPLC] grade; Sigma-Aldrich, Gillingham, UK) and centrifuged at 1,000 × G for 10 min to pellet debris. The supernatant was filtered through a 0.2-µm disposable filter unit (13-mm nylon Puradisc; Whatman, Chalfont St. Giles, UK) into a 2-ml crimp-top HPLC vial (Agilent, Stockport, UK). Diclofenac was separated and quantified by HPLC with electrospray ionization mass spectrometry detection (Agilent) 1100 series equipped with a Waters (Elstree, UK) Xterra MS C18 column and equivalent guard. Diclofenac standard (Sigma-Aldrich; D6899) was dissolved into 1:1 (v/v) acetonitrile:Milli-Q (Millipore UK, Ltd., Durham, UK) water at a 50 mg/l concentration. Calibration standards were then created by dilution of this stock standard in 100% acetonitrile to create a calibration range between 5 and 1,000 µg/l. The calibration was linear across this range with an r^2 value >0.99. Samples and standards (20 µl) were subjected to a binary gradient elution profile, which consisted of 0.1% acetic acid (Sigma-Aldrich) in water (solution A) and in 100% acetonitrile (solution B): starting conditions were 75% A to 25% B for 0.1 min, then a 15-min linear gradient was used from 75%-A:25%-B to 5%-A:95%-B, followed by a 5-min column-wash step using 5%-A:95%-B, and finally a 10-min re-equilibration step with 75%-A:25%-B. Flow rate was 0.7 ml/min, and the column temperature was held at 40 C. The mass spectrometer (Agilent; 1946D) was equipped with an electrospray ionization source, and mass spectra were acquired in the negative ion mode. The mass spectrometer selectively monitored ions 294 and 296 m/z, and 294 m/z (the deprotonated molecular mass) was used to quantify diclofenac residues. The mean diclofenac recovery was 96% in spiked crow plasma and 81% from bovine liver, and the limit of quantification for the method was 10 µg/l in plasma (Taggart et al., 2009).

Pharmacokinetic analysis: Due to the paucity of samples collected as a result of the small size of the Pied Crow (±400 g), complete pharmacokinetic profiles were not obtained. Hence, to obtain an estimate of the average pharmacokinetics of diclofenac in the Pied Crow, the samples collected from individual animals per dose were subjected to naïve average pooling, as described in population pharmacokinetics (Sheiner and Beal, 1981). The single resultant profile was then evaluated using standard equations and a noncompartmental model. To allow comparisons with other bird species, the noncompartmental equations were also fitted to previously published data (Rattner et al., 2008) for Turkey Vultures (*Cathartes aura; n*=4) dosed at either 8 or 25 mg/kg body weight and to sparse data previously presented for a chicken that died when dosed at 5 mg/kg (intramuscular administration; Naidoo et al., 2007; Rattner et al., 2008).

In vitro toxicity assay

Consumables: Collagenase, diclofenac, Dulbecco's modified Eagle's medium (DMEM), dichlorofluorescein diacetate (DCFH-DA), phosphate-buffered saline (PBS), fetal calf serum (FCS), penicillin G, streptomycin, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were all purchased from Sigma South Africa (Johannesburg); culture plates were purchased from NUNC, South Africa (Sandton) and Matrigel® was purchased from the Scientific Group, South Africa (Johannesburg).

Organ harvesting and culture: Three adult crows were caught and immediately euthanized using the procedures described above without administering any treatment or diclofenac. Each crow contributed to a replicate, which was undertaken, 1/wk for 3 wk. Following euthanasia, the kidneys and liver were immediately removed and placed on ice. The organs were subsequently used to ascertain the renal tubular epithelial and hepatic cell sensitivity to diclofenac. Kidney tissue was minced with a scalpel blade and incubated overnight in DMEM with 0.2 mg/ml of collagenase at 37 C (Freshney, 1987). Cell homogenates were then passed through a 250- and 38-μm filter to isolate the renal tubular epithelial (RTE) cells. The cells were washed three times with PBS buffer by centrifugation at 200 × G and resuspended in DMEM supplemented with 10% FCS, streptomycin (50 IU/ml), and penicillin G (50 IU/ml). The RTE cell suspensions (200 μl at 10^5 cells/ml) were seeded into 96-well plates (Lu et al., 2004) and given 48 hr at 37 C in 5% CO_2 to establish before incubation with diclofenac (at 80, 40, 20, and 10 μM concentrations) for an additional 48 hr in the presence or absence of uric acid (0.18 mM). Cultures were then incubated with 0.44 μM MTT for 5 hr to assess cell viability (Gerlier and Thomasset, 1986). The degree of formazan formation was determined on a Varian spectrophotometer (Varian Medical Systems, Inc., Palo Alto, California, USA) at 570 nm (with a 1-cm path length). Cell survival was evaluated as a percentage of viable cells in treated versus untreated control wells on the same plate (% cell viability). The amount of reactive oxygen species (ROS) present was determined in a similar manner, except cultures were incubated with DCFH-DA dye for 30 min, and readings were taken at 504 nm (Gomez-Lechon et al., 2003; Somogyi et al., 2007).

Whole livers were perfused with saline and then with collagenase (0.2 mg/ml) via a hepatic vein until the hepatic architecture became disaggregated. The hepatocytes were then collected by incising the intact liver capsule. Isolated cells were washed three times by centrifugation at 200 × G with PBS buffer and resuspended in DMEM supplemented with 10% FCS, streptomycin (50 IU/ml) and penicillin G (50 IU/ml). The hepatocyte (1 ml; 10^5 cells/ml) suspensions were then seeded into 96-well plates precoated with Matrigel (200 μl; Scientific Group). The 96-well plates were given 48 hr to establish before incubation with diclofenac in the absence or presence of uric acid as above. The ROS and percentage of cell viability were determined the same way as they were for the RTE cells.

RESULTS

No signs of overt toxicity were observed, and no gross lesions were seen in any birds. Clinical pathology parameters were not significantly different between treated and control birds.

Organ and plasma samples were analyzed for their residual concentration of diclofenac. No drug was detectable in the tissues of any of the crows dosed at 0.8 mg/kg or 10 mg/kg. Likewise, no drug residues were detected in the 48-hr plasma samples ($n=2$) following the 10 mg/kg dose, but crows ($n=2$) sampled at 4 and 12 hr had very low plasma concentrations of, on average, 0.1 μg/ml and 0.01 μg/ml, respectively. The estimated pharmacokinetic parameters based on naïve dosed pooling are given in Table 1. The profile was characterized by a short half-life of elimination (2.33 hr) and a small, estimated area under the curve (0.05 μg/ml/hr). In addition, the drug was characterized by an extremely large volume of distribution (58.35 l/kg).

To better understand the absence of toxicity, we evaluated the direct effects of diclofenac on liver and renal tubular cell cultures (Fig. 1). The RTE cell cultures showed very low levels of toxicity (>80% cell viability) when exposed to diclofenac in either the presence or absence of uric acid. In kidney, the toxicity was not dose dependent; in liver, diclofenac produced a dose-related effect in the presence of uric acid, but a nondose-dependent effect in the absence of uric acid. As in the kidneys, the evident toxicity was very low. No increases in percentage of ROS production were observed in any of the experimental wells. However, the dose-responsive toxicity for diclofenac-only hepatocytes (in the absence of uric acid) corresponded with increasing ROS production, which was ameliorated by the addition of uric acid (Table 2). In contrast,

TABLE 1. Estimated pharmacokinetic parameters calculated using noncompartmental modeling for the Pied Crow following administration of diclofenac at 10 mg/kg by oral gavage.

Parameter[a]	Profile 1[b]	Profile 2[b]	Mean	SD
Peak concentration (μg/ml)	0.11	0.11	0.11	0.004
Time of peak (hr)	4.00	4.00	4.00	0.000
Trough concentration (μg/ml)	0.01	0.01	0.01	0.001
Time of trough (hr)	12.00	12.00	12.00	0.000
Dose (mg/kg)	10.00	10.00	10.00	0.000
AUC_{last} (μg/ml/hr)	0.56	0.52	0.54	0.029
AUC_{inf} (μg/ml/hr)	0.60	0.55	0.58	0.036
λz (1/hr)	0.29	0.31	0.30	0.014
AUMC (μg/ml/hr^2)	3.15	2.87	3.01	0.192
$T_{½}$ (hr)	2.41	2.26	2.33	0.107
MRT (hr)	6.21	6.02	6.11	0.133
V_z (l/kg)	57.70	59.00	58.35	0.922
V_{ss} (l/kg)	103.04	109.03	106.03	4.231
Clearance (l/hr/kg)	16.61	18.12	17.36	1.069

[a] AUC_{last} = area under plasma concentration versus time curve to the last time point; AUC_{inf} = area under the plasma concentration versus time curve extrapolated to infinity; λz = elimination constant; AUMC = area under the moment curve; $T_{½}$ = half-life elimination; MRT = mean residence time; V_z = volume of distribution; V_{ss} = volume of distribution at steady state.

[b] The two profiles were created by naïve dose pooling of data from four birds.

the RTE cultures showed no increase in ROS production following treatment (Table 2).

DISCUSSION

The lack of toxicity at both low and higher doses was unexpected. Reported parameters (i.e., UA, urea, ALP, and K^+), which were all significantly altered by diclofenac toxicity in *Gyps* spp. vultures, remained unchanged in crows. Likewise, we observed no evidence of toxicity on necropsy performed 48 hr after the 10 mg/kg dose (i.e., the study was designed to pick up acute changes if they were present). These findings were in marked contrast to vultures, where severe clinical signs of depression were evident 24 hr postexposure and worsened until the birds entered a coma and died around 48 hr postexposure. In addition, these vultures always demonstrated massive increases in serum uric acid concentrations with concurrent visceral gout evident on necropsy. Our findings were, however, very similar to those previously reported for Turkey Vultures (Rattner et al., 2008), suggesting that toxicity is not linked to consumption of carrion (and consequential to high uric acid generation) but to other biochemical factors.

Although it was not possible to obtain full pharmacokinetic profiles for individual birds, parameters were estimated using naïve dose pooling and equations designed for therapeutic dose monitoring (Table 1). Although these parameters are, by nature, likely to be overestimates, they still provide important indications of metabolic capacity and the total extent of exposure and are, therefore, used in an attempt to explain the lack of toxicity, instead of describing the pharmacokinetics of diclofenac in the crow. In the Pied Crow, diclofenac was characterized by a rapid half-life elimination, which was much shorter than that observed in Turkey Vultures and *Gyps* spp. (Swan et al., 2006b; Rattner et al., 2008; Naidoo et al., 2009). The half-life elimination was, however, twice that reported for the Domestic Chicken (as evaluated in a full pharmacokinetic study), which also survived with no signs of toxicity. The crow profile was also characterized by very low plasma

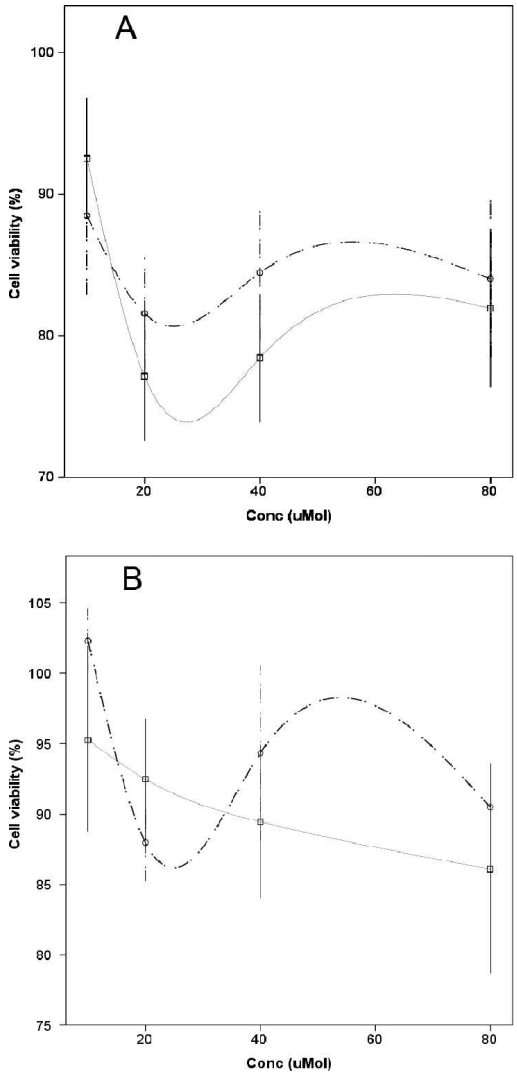

FIGURE 1. Percentage of cell viability for renal tubular (A) epithelial cells and (B) hepatocytes following incubation with diclofenac in the presence (square) of absence (circle) of uric acid, following 48 hr of incubation.

diclofenac concentrations and, in turn, a low level of exposure (0.54 μg/ml per hour), indicating that overall exposure in the systemic circulation was low (reflected by the large volumetric distribution [V_z]). Although parenteral administration is required to provide a better description of the pharmacokinetics, low systemic circulation in conjunction with a large V_z suggests presystemic elimination plays a major role in the oral bioavailability of diclofenac in Pied Crows.

The pharmacokinetic parameters (in comparison to other avian species) demonstrated a clear relationship between half-life and death/toxicity (Table 3; Swan et al., 2006b; Naidoo et al., 2007, 2009; Rattner et al., 2008). In all cases where plasma concentration data were available, birds tended to die when half-life elimination was above 12 hr. However, when the half-life was <7 hr, animals survived. This observation was marked in the chicken, where a low dose resulted in no mortality and showed a half-life of 1 hr, whereas, at a 5 mg/kg dose, the one bird treated died and displayed a half-life profile of 14 hr. This suggests that toxicity is related to zero-order metabolism. A similar finding was reported in a pharmacokinetic study of ketoprofen in Cape Griffon Vultures (*Gyps coprotheres*), where toxicity occurred at higher doses, when half-life elimination also indicated zero-order metabolism (Naidoo et al., 2010a). Zero-order pharmacokinetics implies that the liver (or a related organ of metabolism) fails to adequately metabolize the drug in a timely manner. Because of this limitation, the drug accumulates within the body until toxicity occurs.

To determine whether diclofenac toxicity was related to half-life elimination, and not to cellular sensitivity (as seen in *Gyps* vultures), isolated renal tubular and hepatic cells were exposed to four concentrations of diclofenac for 48 hr. The long period of exposure was used to simulate an extremely long mean residence time in vivo, which corresponded to the maximum incubation period observed in other studies (Ng et al., 2008; Naidoo et al., 2009). In addition, the concentrations used were significantly larger than those actual concentrations measured in the crow at 4 hr in the pharmacokinetic profile. In hepatocytes, toxic effects were minimal and cell viability fluctuated between 80 and 100%. A linear toxic-response relationship was also present in the diclofenac group in the

TABLE 2. Reactive oxygen species formation, as a percentage of the control, for renal tubular epithelial cells and hepatocytes incubated in the absence or presence of uric acid.

	Uric Acid Absent		Uric Acid Present	
Diclofenac (μMol)	Kidney	Liver	Kidney	Liver
5	98.16	110.05	97.52	103.72
10	94.63	108.20	98.72	104.66
20	96.51	98.96	98.69	104.23
40	98.54	91.88	100.54	106.26

presence of uric acid, which corresponded with increased ROS production, as seen in chicken RTE cells (Naidoo et al., 2009).

As with the hepatocytes, RTE cells were also minimally affected by the presence of diclofenac (>80% cell viability observed), with the evident toxicity being unrelated to ROS production. The low level of toxicity was similar to that reported by Ng et al. (2008), where mammalian cells were also minimally sensitive to the toxic effects of diclofenac. However, Pied Crow cells showed markedly different results from those of chickens, where cell viability was <20% following a similar period of incubation (Naidoo and Swan, 2009). This indicates that the RTE cells of the Pied Crow are even more tolerant to diclofenac and that rapid hepatic metabolism is not the only reason why crows are insensitive to diclofenac. It may even be possible that the CYP2c9 (cytochrome P450 2C9) concentrations within the crow RTE cells are naturally higher, thereby offering a greater degree of protection; the rapid metabolism may be due to a combined effect caused by liver and extrahepatic kidney metabolism.

In summary, if we consider that slow diclofenac metabolism in bird species is linked to toxicity, we suggest that rapid metabolism (and thus low systemic bioavailability) may indicate tolerance to diclofenac (as noted in Pied Crows and Turkey Vultures). In addition, toxicity seems to be clearly related to the sensitivity of the RTE cells to diclofenac. This is demonstrated by the minimal toxicity in primary crow RTE cells, as opposed to chicken RTE cells, despite exposure at equal concentrations. The Pied Crow is, therefore, not a suitable model for the testing of other nonsteroidal

TABLE 3. Estimated selected pharmacokinetic parameters for avian species dosed with diclofenac.[a]

Species	Common name	*n*	Route	Dose[b] (mg/kg)	Status	$T_{½}$ (hr)	Cl (l/hr/kg)	AUC (μg/ml/hr)	Reference
Gallus gallus	Chicken	6	Oral	0.8	Alive	0.89	0.189	4.33	Naidoo et al., 2007
Corvus albus	Pied Crow	2	Oral	10	Alive	2.33	17.36	0.54	the present study
Cathartes aura	Turkey Vulture	2	Oral	25	Alive	6.29	0.26	141.15	Rattner et al.,
Cathartes aura	Turkey Vulture	2	Oral	8	Alive	6.43	0.79	13.86	2008
Gyps coprotheres	Cape Griffon Vulture	2	Oral	0.8	Dead	12.24	0.01	80.28	Naidoo et al., 2009
Gallus gallus	Domestic Chicken	1	Im	5	Dead	14.34	0.65	6.30	Naidoo et al., 2007
Gyps africanus	African White-backed Vulture	2	Oral	0.8	Dead	16.78	0.02	61.95	Naidoo et al., 2007

[a] $T_{½}$ = half-life elimination; Cl = plasma clearance; AUC = area under the plasma concentration versus time curve; Im = intramuscular.

[b] Values for all species, except *G. domesticus* at 0.8 mg/kg oral route, were estimated using equations described for noncompartmental modeling.

anti-inflammatory drugs for overt toxic effect.

ACKNOWLEDGMENTS

This study was approved by the Animal Use and Care Committee of the University of Pretoria. Crow capture was approved by the North-West Department of Conservation. The study was partially funded by the Royal Society for the Protection of Birds (RSPB), The Worldwide Fund for Nature (WWF), and the National Research Foundation's (NRF) Thutuka program. Many thanks to K. Wolter, E. Kilian, and A. Venter for technical support.

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Submitted for publication 28 July 2010.
Accepted 12 April 2011.