CHAPTER 1: Introduction

1.1 The Vulture Crisis

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

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

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

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

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

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

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

1.2 Hypotheses

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

1.3 Objectives

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

CHAPTER 2: Literature Review

2.1 Vultures: Twenty-first century outcasts

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

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

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

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

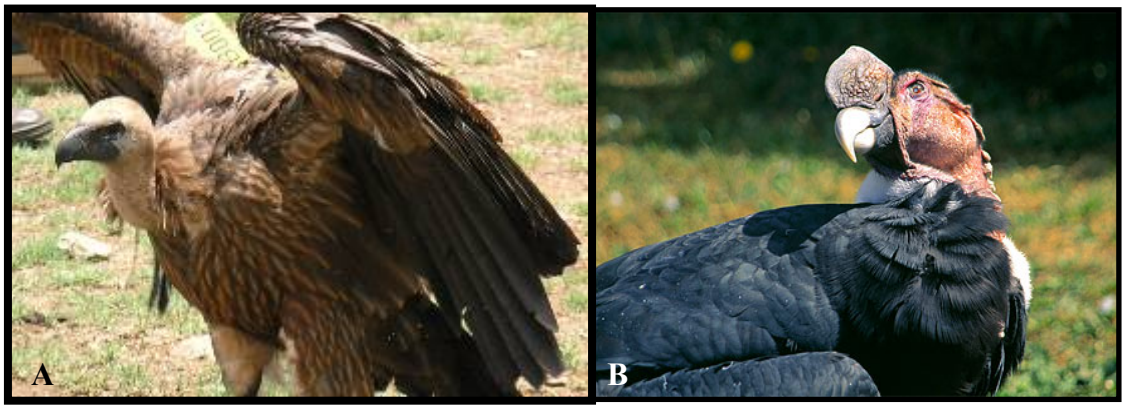

Figure 2-1: The two major branches of the vulture family
A-An old world griffon vulture (*Gyps africanus*) (Picure taken at Licthenberg),
B- A new world vulture (*Vultur gryphus*)(Picture from National Geographic)[69]

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

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

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

A- A gold vulture representing the upper kingdom on the death mask of the child-king Tutankhamun (Picture obtained from the Science Museum)[2],

B- The evil Ravana defeating the vulture king following the battle described in the Ramayana (Picture obtained from wikipedia)[3]

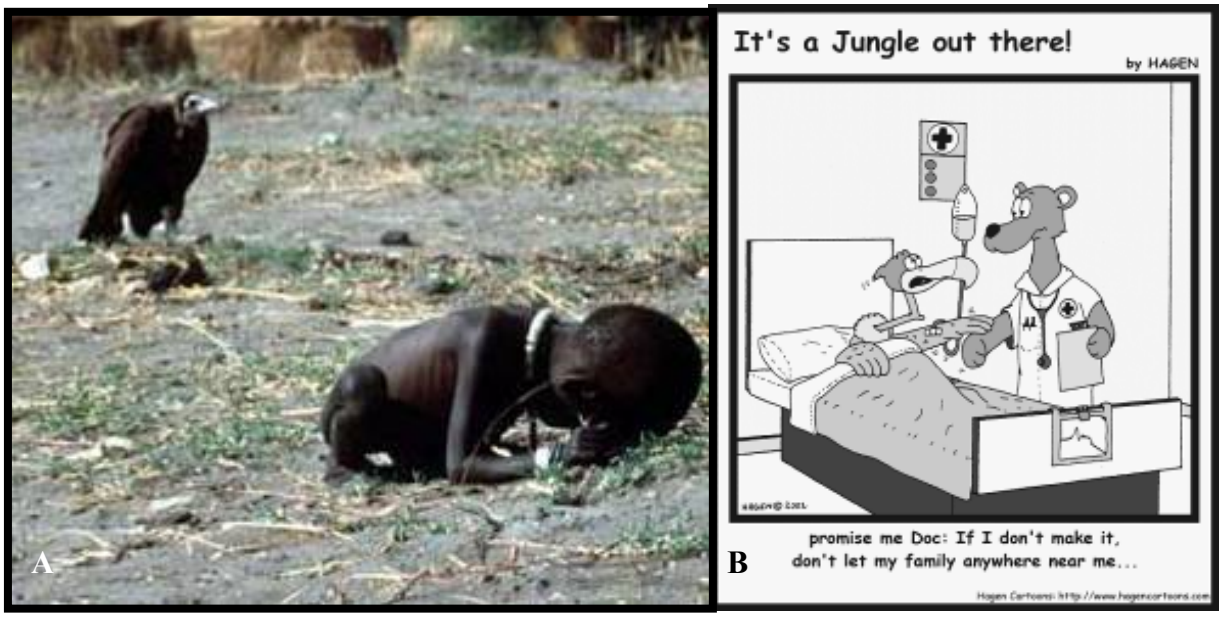

Figure 2-3: Vulture being shown as evil creatures, interested in only death, by popular press and cartoons

A- Pulitzer prized picture taken by photojournalist Kevin Carter, showing a vulture hovering over a weak girl taken in Sudan (1993). Not shown in the picture was that the weak girl walked away after the picture was taken[9]

B- A vulture cartoon portraying the birds as nothing more than cannibals.[55]

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

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

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

2.2 A crash in the Indian Vulture Population

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

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

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

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

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

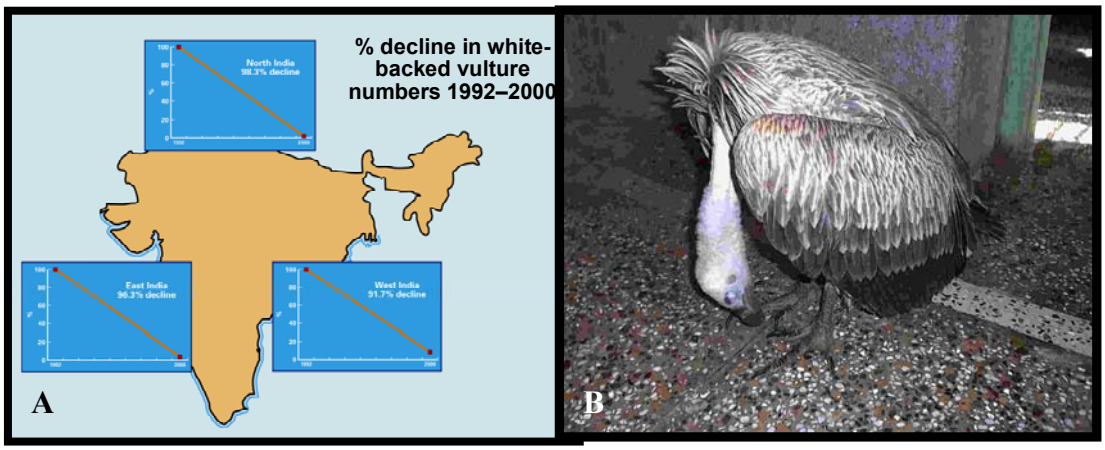

Figure 2-6: Illustration of the catastrophic decline in the vulture numbers (Courtesy of the RSPB)

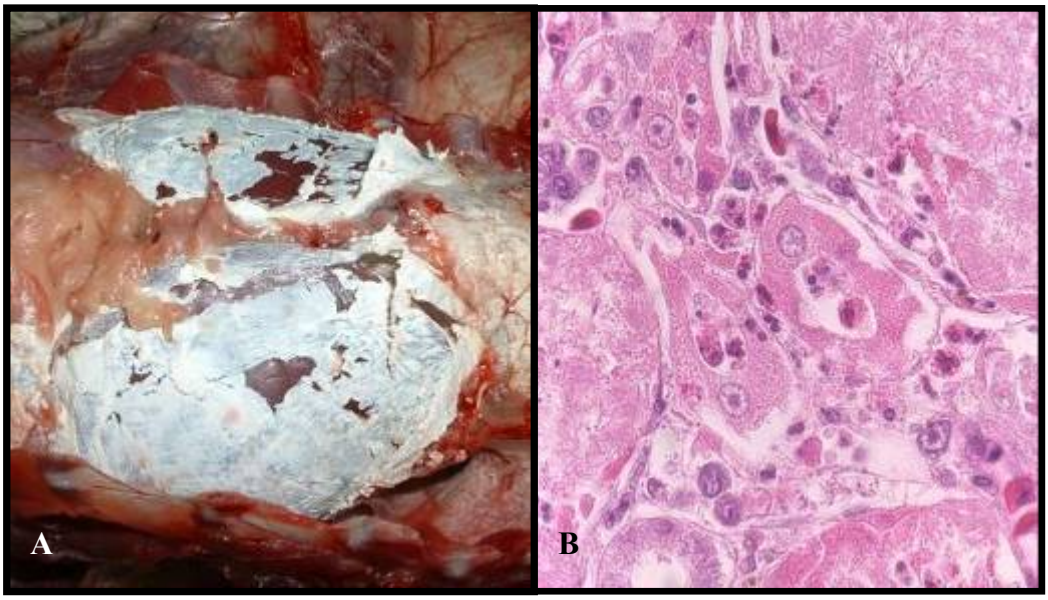

Figure 2-7: Typical necropsy and histopathological lesions seen in the poisoned birds

A- Diffuse pasty white material covering the abdominal viscera (Pictures courtesy of Prof L Oaks

B- Histopathological lesion showing renal tubular damage (Pictures courtesy of Prof L Oaks)

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

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

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

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

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

2.2.2 *Why were the birds exposed to diclofenac?*

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

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

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

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

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

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

2.3 Impact of a Declining Vulture Population

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

2.3.1 *Aesthetic value*

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

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

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

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

2.3.2 *Importance to the Parsi Community*

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

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

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

2.3.3 *Increase in the dog population*

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

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

2.3.4 *Loss of income to the bone collectors*

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

2.3.5 *Air travel*

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

2.4 NSAIDs: An Overview

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

2.4.1 *Chemistry*

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

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

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

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

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

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

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

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

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

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

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

2.4.2 *Mechanism of Action*

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

2.4.2.1 COX mediated effects

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

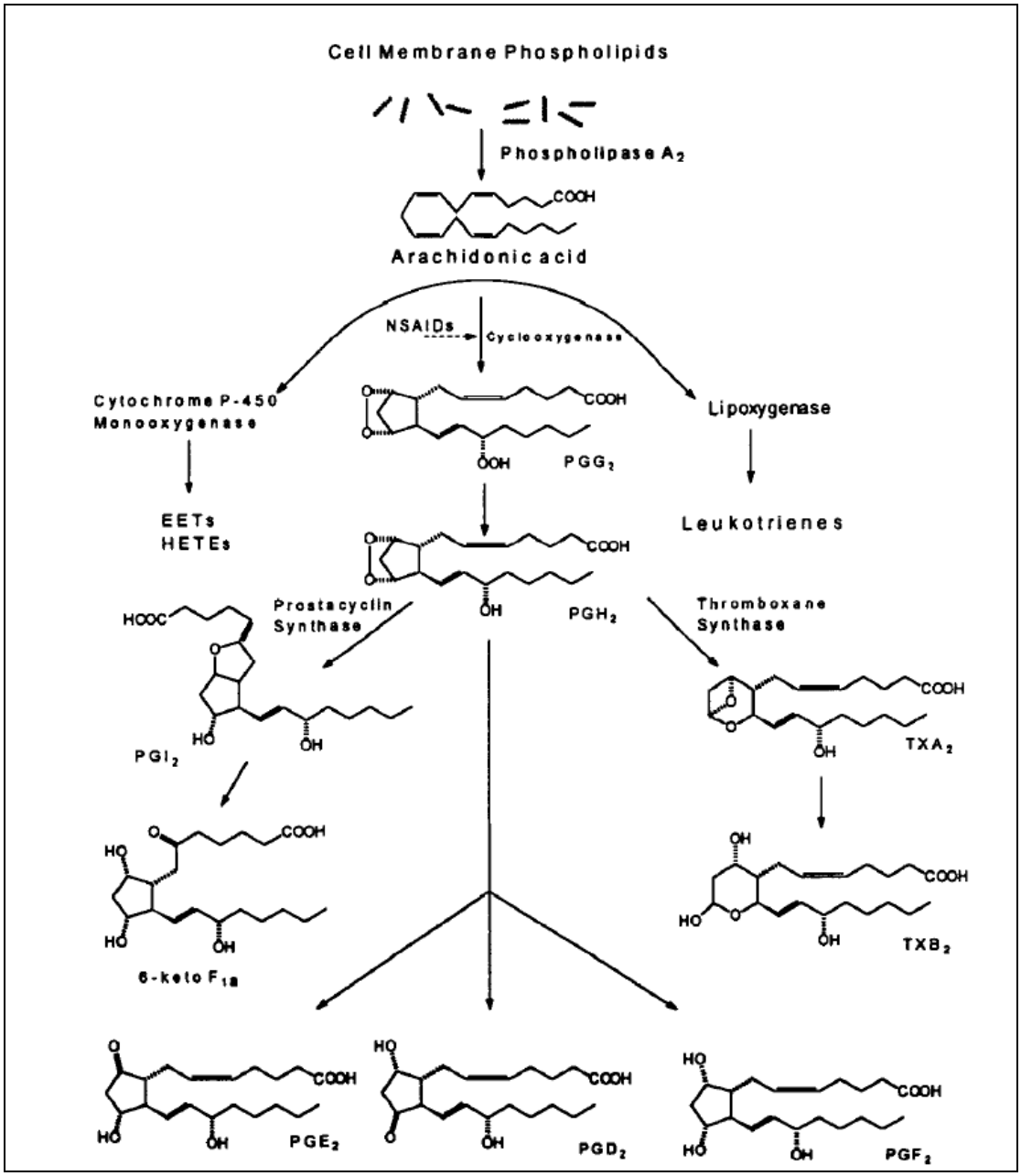

Figure 2-8: Metabolic pathways of the arachidonic acid cascade. NSAIDs: Non-steroidal anti-inflammator drugs; EETs: epoxyeicosatrienoiac acid; HETEs: hydroxyeicosatetraenoaic acids; PG: prostaglandin; TX: thromboxane[96]

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

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

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

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

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

2.4.2.2 Non-COX mediated Effects

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

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

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

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

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

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

2.4.3 *Pharmacological Activity*

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

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

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

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

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

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

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

2.4.4 *Adverse Drug Reactions*

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

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

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

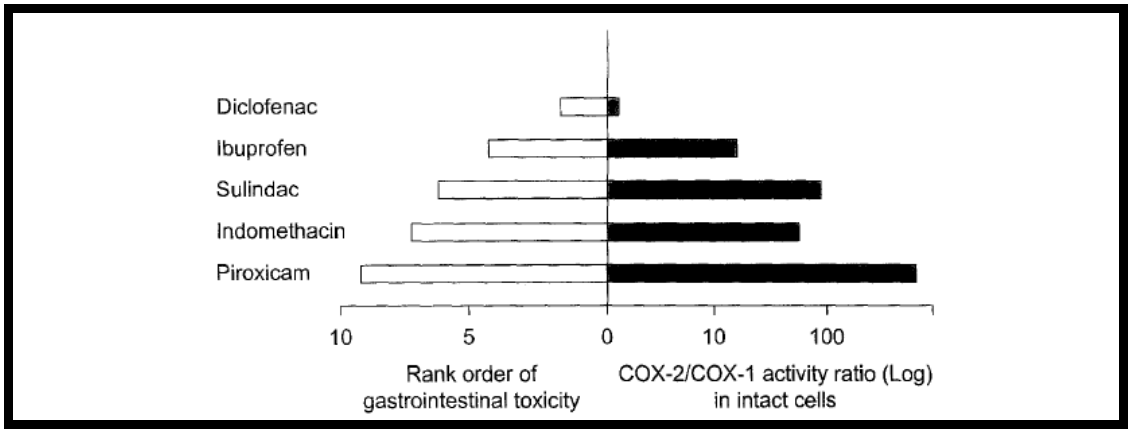


Figure 2-9: Comparison of gastric damage and COX selectivity of various NSAIDs used in people[96].

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

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

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

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

2.4.5 *Duration of Effect*

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

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

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

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

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

2.5 Diclofenac

2.5.1 *Properties*

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

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

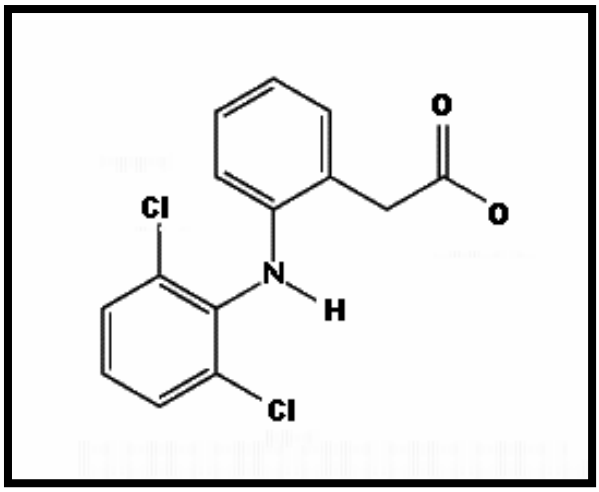


Figure 2-10: Molecular structure of diclofenac

2.5.2 *Possible mechanisms of toxicity in vultures*

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

2.5.2.1 Ischaemic nephropathy with secondary visceral gout

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

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

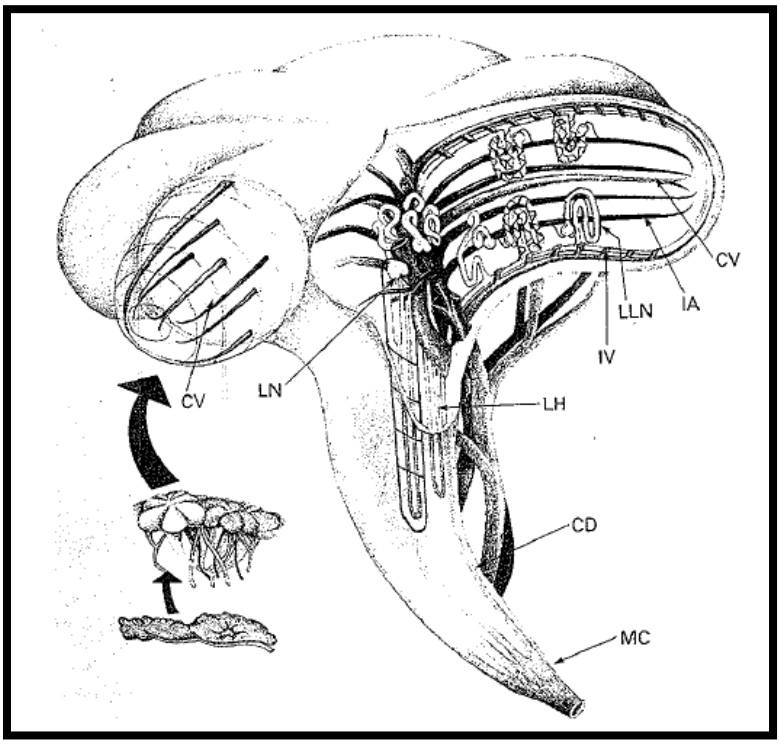


Figure 2-11: Illustration of the two different types of nephrons present in the avian kidney.
Central vein (CV); Loopless nephron (LLN); looped nephrons (LN); loops of Henle (LH); collecting ducts (CD); medullary cones (MC); intralobular arteries (IA), interlobular/afferent veins (IV) [21]

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

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

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

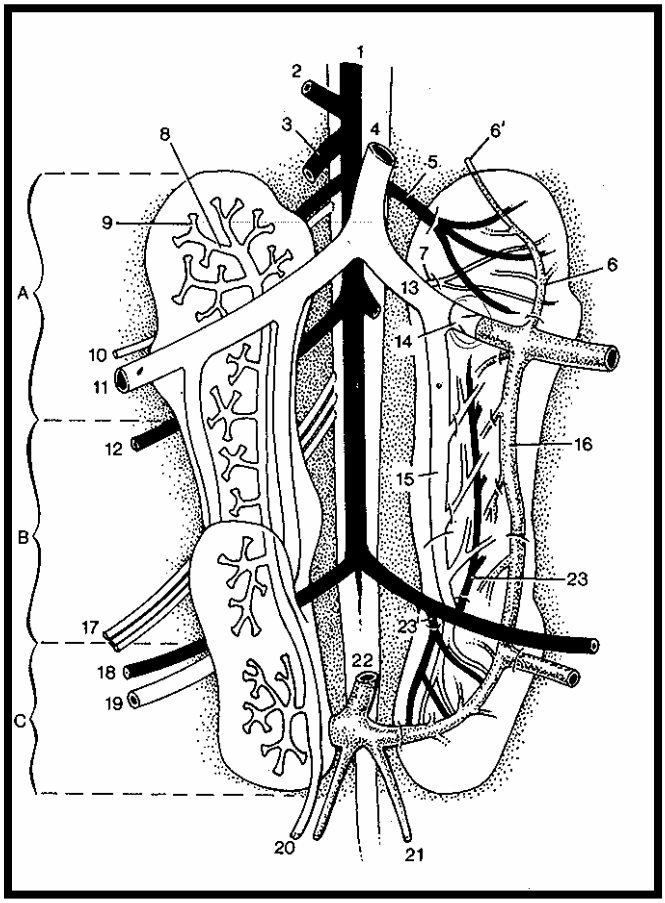


Figure 2-12: Illustration of the dual blood supply to the avian kidney
The right kidney shows the branches of the ureter; the left, the renal vessels. A, B, C, cranial, middle, and caudal divisions of kidney. 1, Aorta, 2, celiac a., 3, cranial mesenteric a.; 4, caudal vena cava; 5, cranial renal a.;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

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

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

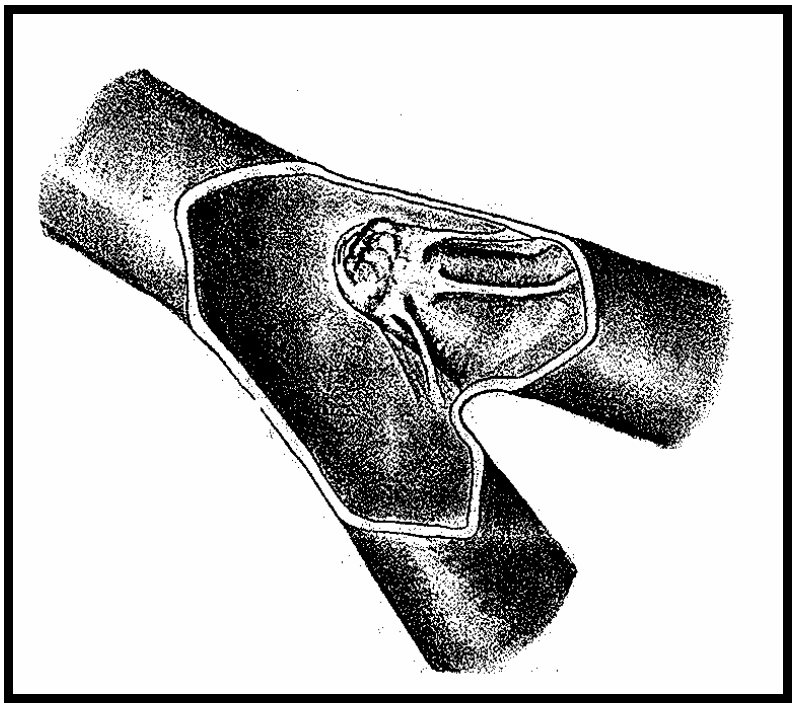
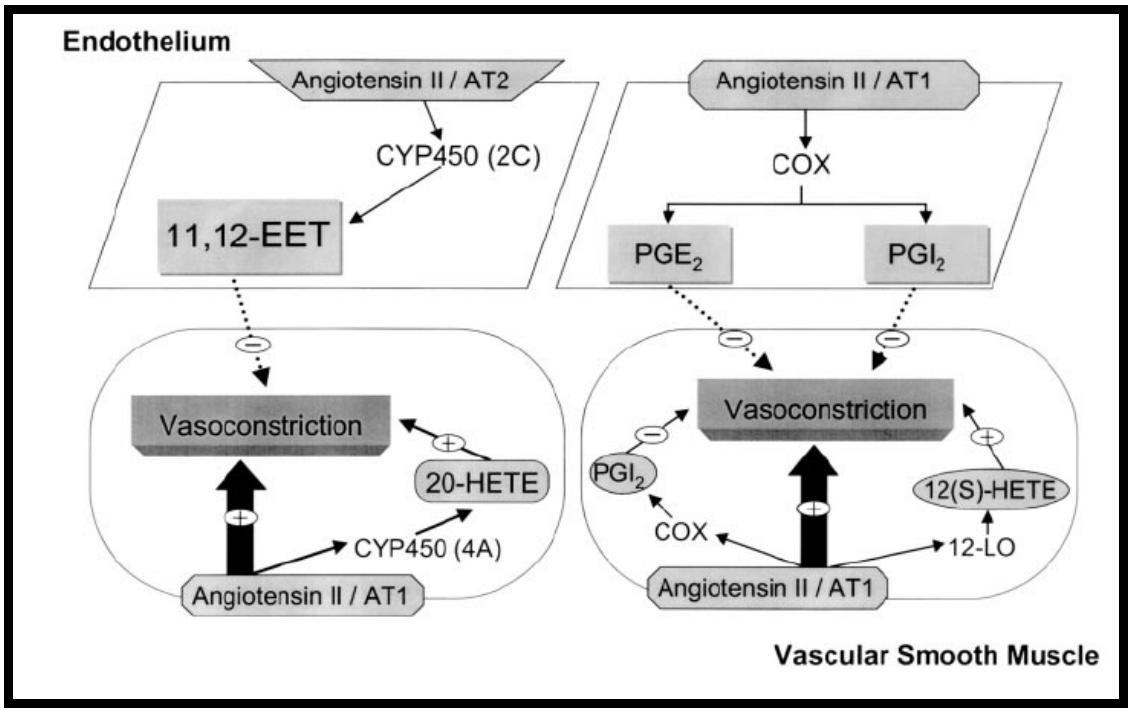


Figure 2-13: Illustration of the conical renal portal valve anchored to the mucosa by cordae tendinae[24]

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

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



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

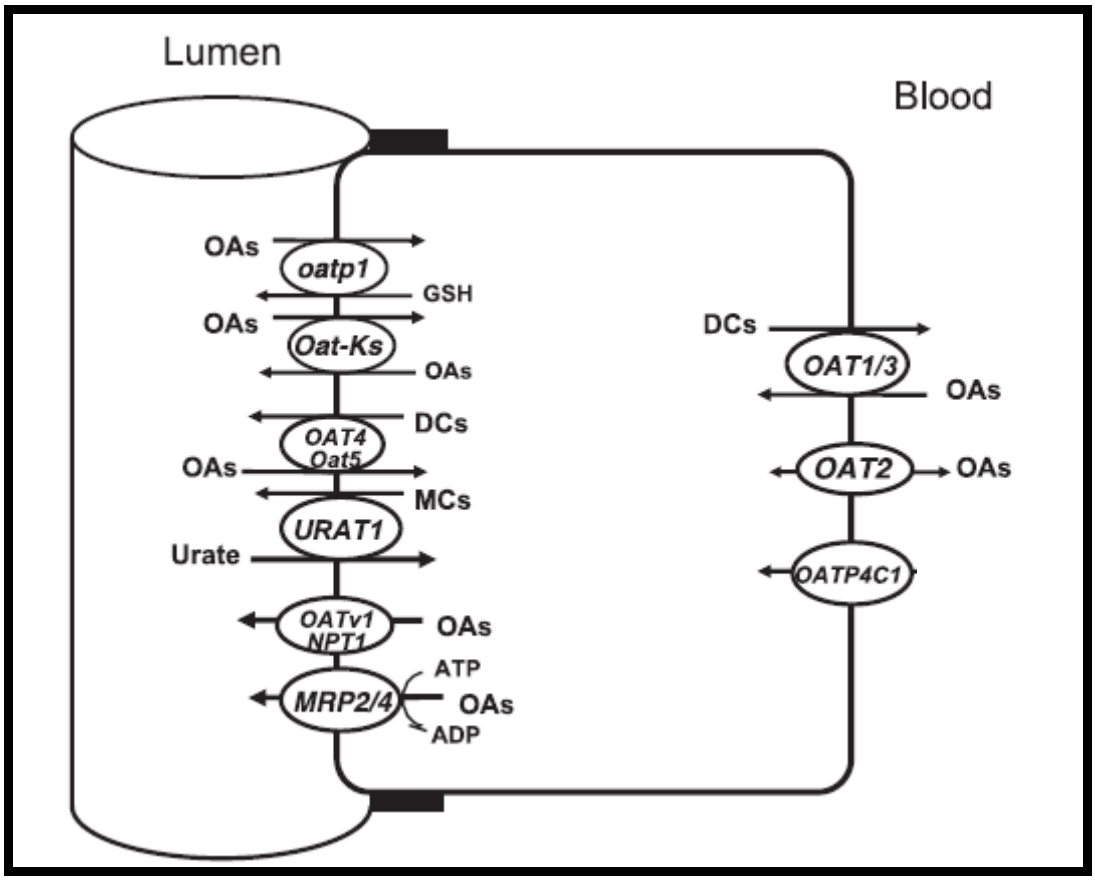
Figure 2-14: Illustration of the prostaglandin linked release following the stimulation of angiotensin receptors by AT2

2.5.2.2 Organic Anion Transporter antagonism

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

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

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



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

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

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

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

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

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

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

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

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

2.5.2.3 Secondary renal toxicity with or without toxic activation

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

2.5.2.3.1 Liver toxicity

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

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

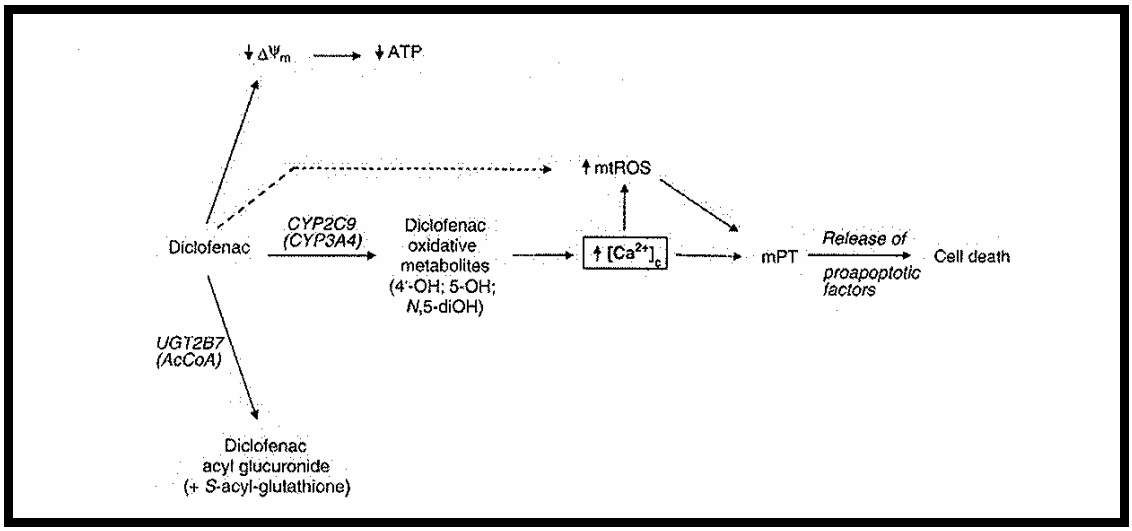


Figure 2-16: Illustration on how the metabolic activation of diclofenac leads to mitochondrial damage and apoptosis (Modified[80])

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

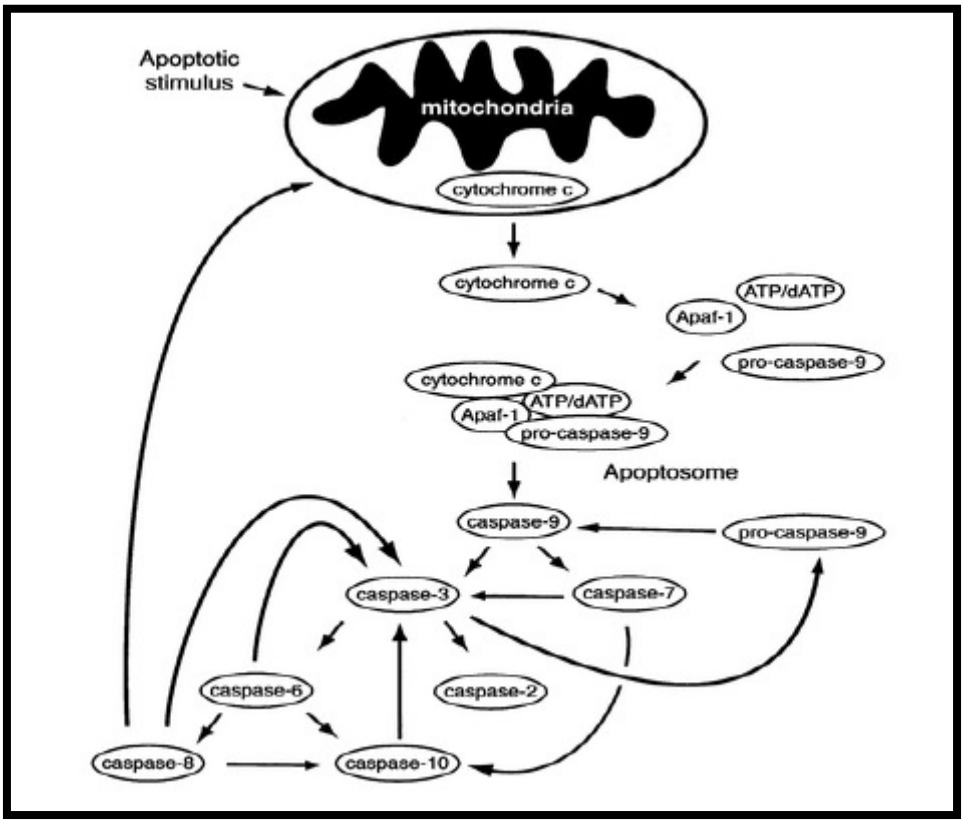
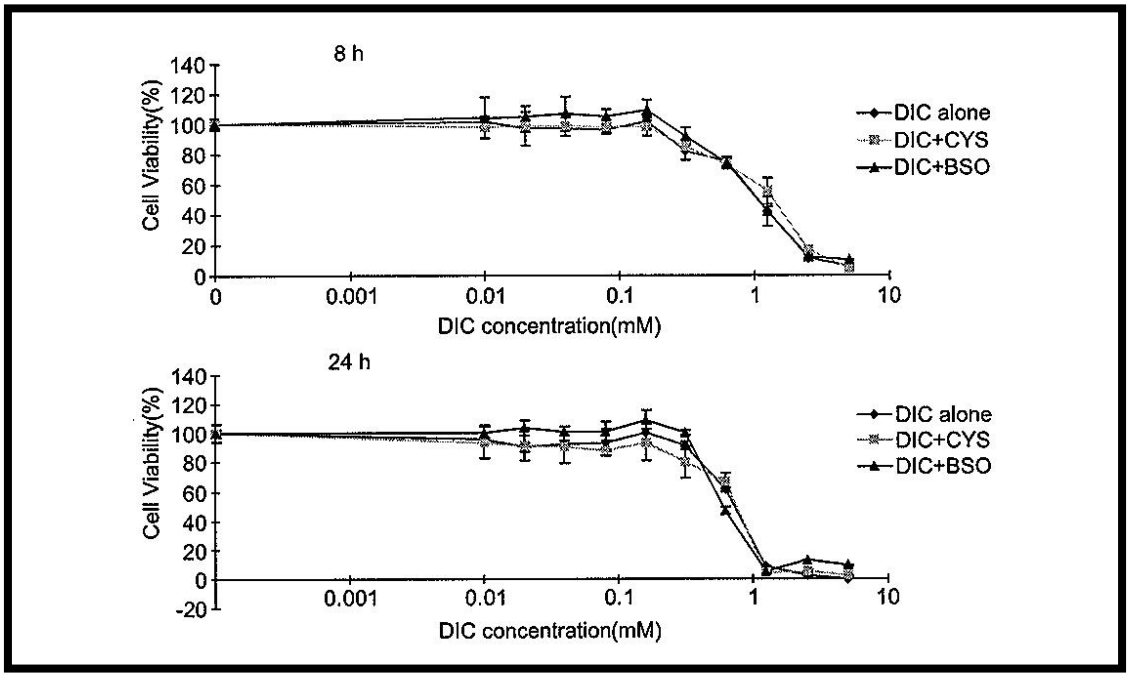


Figure 2-17: The pathways and enzymes involved in the activation of the caspase pathway and cellular apoptosis. Toxicity starts with the release of cytochrome C by the mitochondria with subsequent activation of the caspase pro-enzymes present in the cytoplasm[16]

2.5.2.3.2 Nephrotoxicity

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



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

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

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

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

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

2.6 Towards the protection of a disappearing species

2.6.1 *Steps necessary to protect the species*

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

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

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

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

2.6.2 *Conservation Efforts: Establishment of a captive population*

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

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

2.6.3 *Removal of diclofenac from the food chain*

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

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

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

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

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

Modified from Cuthbert *et al.*, 2006

2.6.4 *The safety of other NSAIDs*

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

2.7 Conclusion

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

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

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