TOXICITY PROBLEMS ASSOCIATED WITH DICLOFENAC IN AVIAN SPECIES AND ITS SUBSTITUTE

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I

DEDICATE

THIS HUMBLE EFFORT

TO

“My Beloved Parents”
ACKNOWLEDGEMENTS

I would like to pay all my praises and humblest thanks to Almighty ALLAH, who bestowed me with the potential and ability to make a material contribution to an already existing ocean of knowledge, and for enabling me to complete this work. I offer my humblest gratitude from my heart to THE HOLY PROPHET “MUHAMMAD” (P.B.U.H.) who is forever a torch of guidance and knowledge for humanity as a whole.

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Finally, the errors contained herein are mine alone.

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ABBREVIATIONS USED:

AAEP: American Association of Equine Practitioners
ALP: Alkaline phosphatase
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
COX: Cyclooxygenase
DDE: Dichlorodiphenyldichloroethylene
DDT: Dichlorodiphenyltrichloroethane
DF: Diclofenac
EC: Environment control
FM: Flunixin meglumine
IM: Intramuscular
IUCN: International Union for Conservation of Nature
KP: Ketoprofen
LPS: Lipopolysaccharide
MTZ: Metamizole
MX: Meloxicam
NS: Normal saline
NSAIDs: Non-steroidal anti-inflammatory drugs
PBZ: Phenylbutazone,
PGs: Prostaglandins
PNS: Physiological normal saline solution
PX: Piroxicam
SD: Standard deviation
TLC: Total leukocyte count
TNFα: Tumor necrosis factor-alpha
U/L: Units per liter
Non-steroidal anti-inflammatory drugs (NSAIDs) are a group of chemically heterogeneous compounds, mostly organic acids, which share certain therapeutic actions and adverse effects. These compounds are divided into two categories based on their activity; nonselective cyclooxygenase (COX) inhibitors and selective COX-2 inhibitors. The first category includes salicylic acid derivatives (aspirin, sodium salicylate), para-aminophenol derivatives (acetaminophen), indole and indene acetic acids (indomethacin, sulindac), heteroaryl acetic acids (diclofenac, ketorolac), arylpropionic acids (ibuprofen, naproxen, ketoprofen), anthranilic acids (fenamates; mefenamic acid, meclofenamic acid), enolic acids (oxicams; piroxicam, meloxicam), pyrazolones (phenylbutazone, dipyrone), nicotinic acid derivatives (flunixin) and alkanones (nabumetone). The second category consists of compounds like diaryl-substituted furanones (rofecoxib), diaryl-substituted pyrazoles (celecoxib) indole acetic acids (etodolac) and sulfonanilides (nimesulide). Aspirin, derived from *Spiraea* and *Salix* plant species, is the progenitor NSAID, which is why the whole class of drugs is known as “aspirin-like drugs”.

All the compounds belonging to this group act by inhibiting the biosynthesis of prostaglandins (PGs) within the cell. A fatty acid, arachidonic acid, is released into the cell from damaged cell membranes to be converted into prostaglandins by the action of various enzymes including cyclooxygenase (COX). These prostaglandins are responsible for the development of inflammation, pain and fever. Cyclooxygenase enzyme has two isozymes i.e. COX-1 and COX-2. COX-1 is constitutively expressed and regulates the synthesis of normally required prostaglandins whilst COX-2 is inducible and produced usually as a result
of tissue insult. Higher concentrations of this isozyme lead to the production of prostaglandins responsible for clinical situations involving inflammation, pain and fever (Simmons et al., 2004). Therefore, the therapeutic efficacy and adverse effects of NSAIDs are attributable to their ability to inhibit prostaglandin formation. The vast majority of NSAIDs is organic acids and, in contrast to aspirin, acts as reversible, competitive inhibitors of COX activity.

In human beings, NSAIDs are used as analgesic, antipyretic and anti-inflammatory drugs. These drugs are used to manage the pain of mild to moderate intensity such as dental and postoperative pain. NSAIDs also lower the body temperature in febrile states. These drugs have their major clinical application as anti-inflammatory agents in the treatment of musculoskeletal disorders such as rheumatoid arthritis, osteoarthritis and ankylosing spondylitis. Other clinical conditions treated with NSAIDs are patent ductus arteriosus in neonates, primary dysmenorrhea in women, systemic mastocytosis, humoral hypercalcemia associated with some neoplasms, Bartter’s syndrome, and hyper prostaglandin E syndrome. NSAIDs are also used in prevention of colon cancer (Anne et al., 2005). In animals, NSAIDs are used to manage pain of musculoskeletal origin, endotoxemia, pyrexia associated with infectious diseases and inflammatory conditions such as rheumatoid arthritis, osteoarthritis, mastitis and canine urinary neoplasia (Lees, 2009).

The major adverse effect of NSAID therapy in humans is the propensity to induce gastric or intestinal ulceration and bleeding. It is estimated that the patients using nonselective NSAIDs have three times greater risk of serious adverse gastrointestinal (GI) events compared to nonusers (Gabriel et al, 1991). Back diffusion of acid into gastric mucosa is one of the causes of GI damage. Long-term parenteral administration of NSAIDs can lead to gastric damage
and bleeding. This effect may be brought about by the inhibition of biosynthesis of cytoprotective PGs, especially PGI-2 and PGE-2 in the gastric mucosa. These PGs inhibit gastric acid secretion, enhance mucosal blood flow and promote cytoprotective mucus production in the intestine (Mizuno et al., 1997).

Other side effects of NSAIDs reported in human beings are disturbances in platelet function, prolongation in gestation or spontaneous labor, premature closure of patent ductus and changes in renal function. In animals, reported adverse effects of NSAIDs are involvement of GI, hematopoietic and renal systems, and are attributable to the inhibitory effects of these drugs on PG activity. Like humans, GI damage is the most common and serious adverse effect in many animals. Gastroduodenal erosions and ulceration reflect the inhibition of PGE2-mediated bicarbonate and mucus secretion, epithelialization and blood flow. Local irritation by the acidic drugs, especially salicylates, injures mucosal cells and submucosal capillaries, and impaired platelet activity may contribute to mucosal bleeding. Oral ulceration due to phenylbutazone is reported in horses. NSAIDs also inhibit platelet aggregation and prolong the clotting time. Analgesic nephropathy is more common in people than animals. This situation occurs due to the inhibition of vasodilatory PGs which ensure medullary vasodilation and urinary output, resulting in renal arterial vasoconstriction (Boothe, 2001).

Diclofenac, one of the important NSAIDs, is a phenyl acetic acid derivative. It was developed especially as an anti-inflammatory agent. It has good analgesic, antipyretic and anti-inflammatory activity. It is categorized as a nonselective COX inhibitor with potency substantially greater than that of indomethacin and naproxen. In addition, diclofenac appears to reduce intracellular concentrations of free arachidonate in leukocytes, perhaps by altering release or uptake of the fatty acid. In humans, diclofenac is rapidly and completely absorbed
after oral administration, and the peak plasma concentration is reached within 2-3 hours (Reiss et al., 1978). There is a substantial first pass effect, and approximately 50% of the drug is available systemically. In humans, diclofenac extensively binds to plasma proteins (99%), and its plasma half-life ranges from 1 to 2 hours. It is metabolized in the liver by a cytochrome P450 isozyme of CYP2C subfamily to 4-hydroxydiclofenac, the principal metabolite and other hydroxylated forms. After glucuronidation and sulfation, the metabolites are excreted in urine (65%) and bile (35%). Diclofenac sodium is recommended for the long-term, symptomatic treatment of rheumatoid arthritis, osteoarthritis and ankylosing spondylitis in people. It is also employed as the short-term treatment of acute musculoskeletal injury, acute painful shoulder, postoperative pain and dysmenorrhea. Addition of mesoprostol, a PGE analog, reduces the frequency of gastrointestinal ulcers and erosions whilst retaining the efficacy of diclofenac in humans (Morant et al., 2002).

Postoperative inflammation after cataract extraction is treated with an ophthalmic solution of diclofenac. GI damage like ulceration, bleeding or perforation of the intestinal wall is the adverse effect of diclofenac in human beings. Elevated hepatic aminotransferase activity indicates the possibility of liver damage by this drug. The probability of liver damage is also supported by various animal studies (Aydin, et al., 2003). Other adverse effects associated with diclofenac therapy include CNS effects, skin rashes, allergic reactions, fluid retention, edema and rarely the impairment of renal function. Diclofenac is not recommended in children, nursing mothers and pregnant women. Although the use of diclofenac in animals is not recommended in the USA and many European countries, its use was initiated in South Asian countries including India, Pakistan, Nepal, Sri Lanka and Bangladesh during the last decade (Risebrough, 2004; Green et al., 2007).
Diclofenac was used in domestic animals to treat clinical conditions like arthritis, lameness, and colic. Diclofenac was also used as an adjunct therapy of infectious diseases to alleviate the signs of pain, inflammation, and pyrexia. No major side effects of this drug have been reported in domestic animals. However, diclofenac has been held responsible for the catastrophic decline in the vulture population in the region. The decline in the number of vultures was first reported in Koleadeo National Park in Bharatpur, Rajasthan, India (Prakash, 1999). The countrywide surveys conducted later in India, showed that 97% of the population of white-backed vultures (Gyps bengalensis) was lost over a period of twelve years, while the other species were less affected (Prakash et al., 2003). A similar decline in vulture populations was also reported from Punjab province of Pakistan (Gilbert et al., 2002). This situation led to ecological imbalances.

Keeping in view the importance of vultures in the ecosystem, many international organizations including The Peregrine Fund, WWF and Birdlife International got involved to investigate and solve this issue. Vultures are natural scavengers and clean up the environment, including through removal of the carcasses of domestic and wild ungulates. Moreover, the meat of cattle and some other animal species is not being used by the people of the region due to certain social and cultural norms. Hence these animals, after death were used by the vultures. In the absence of vultures these carcasses remain in place for comparatively longer periods of time, putrefying and serve as the potential source of contagious diseases for other animals and the people as well. The problem was deeply studied by zoologists, who postulated that a variety of reasons were responsible for the decline in vulture population, including infectious disease outbreak, intentional or accidental poisoning, persecution by the people, loss of natural habitat due to deforestation, and
shortage of food. The investigators categorically ruled out all the proposed causes except the possibility of the presence of some chemical compound in their food chain. After detailed investigations, the researchers came to recognize that diclofenac, a pharmaceutical product being used to treat domestic animals for various indications, was the major cause of the huge causalities in the vultures. They proposed that diclofenac residues present in carcasses of animals that had been treated shortly before death, when consumed by the vultures, caused renal damage leading to the development of a syndrome, ‘visceral gout’. The birds being uricotelic, excrete uric acid as the end product of nitrogenous compound metabolism. With renal damage in birds there can be an impairment of the normal excretion of uric acid from body, such that it starts crystallizing on the surfaces and inside the visceral organs resulting in visceral gout and ultimately death. Later, the scientists reproduced the similar disease in vultures after the administration of diclofenac to them. The findings of these studies indicated that the vultures were highly sensitive to the toxicity of diclofenac, even at very low doses (0.8 mg/kg). Also, vultures experienced serious effects of the drug after being fed the meat of buffalo and goat that had received therapeutic doses of diclofenac(Oaks et al., 2004: Shultz et al., 2004). Based on the observations of these studies, the regional governments, i.e. India, Pakistan, and Nepal imposed a ban on the use of diclofenac in animals in 2008. To make the ban enforceable, there was a dire need to find a safe alternate NSAID for use in veterinary practice. Such replacement was necessary to avoid depriving the animal patients and practicing veterinarians of an efficacious NSAID. Hence, the present research project was designed with the following objectives in mind;
1. To develop a surrogate model to study the toxicity of NSAIDs including diclofenac using broiler chickens as an alternative to the vulture, keeping in view the availability and management challenges with the latter.

2. To study the toxicity of diclofenac in different bird species.

3. To screen various NSAIDs being used in human and veterinary medicine for their safety profile in experimental birds and to find a safe alternative to diclofenac.

4. To study the comparative efficacy of safer alternate candidate NSAIDs in target species under controlled as well as clinical situations.

A study was designed to develop the chicken experimental model in which the toxicosis resembled that in vultures, including similar signs and lesions. Later, a series of five experiments was conducted to meet the listed objectives. In the first experiment, the toxicity of diclofenac was studied in broiler chicken following oral and intramuscular administration. The second experiment was planned to compare the toxicity of this drug in different avian species, including chickens, pigeons, and quail. A number of different NSAIDs being used in human and veterinary medicine were screened for their safety profile in experimental chickens in the third experiment. The NSAIDs found safer in these experiments were evaluated for comparative efficacy in domestic animals in experimental and field conditions.
Chapter 2

REVIEW OF LITERATURE

Statement of the problem

Non-steroidal anti-inflammatory drugs (NSAIDs) belong to a class of compounds used to treat conditions such as inflammation, fever, and pain in humans and animals. Diclofenac is a NSAID, which is extensively used in humans for the treatment of arthritis, to manage postoperative, dental, and other forms of pain, as well as fever. The use of this drug was started in veterinary practice during the last decade in Southeast Asian countries, and such usage became widely available in the region (Risebrough, 2004). Recently it was reported that diclofenac residues caused a catastrophic decline in the vulture population when they consumed the meat of treated livestock carcasses (Oaks et al., 2004; Shultz et al., 2004). Now at least three species of Gyps vultures are listed as, ‘Critically Endangered’ (IUCN, 2006). Vultures are natural scavengers and remove the carrions of livestock and wild ungulates, thereby cleaning decaying animal carcasses from the environment. In the absence of vultures there remains a potential threat of spread of infectious diseases from putrefying carcasses to livestock, wildlife, and humans. There may be an increased risk of spread of rabies through dog bite in humans and animals as there is evidence of a rise in the number of this species due to an increased food supply after the disappearance of vultures (Pain et al., 2002).

Keeping in view the importance of vultures as an integral part of ecosystems, many organizations came forward to investigate the reasons of decline and formulate relevant conservation strategies. In this situation, it was important to assess the toxicity of diclofenac
utilizing an avian experimental model and to investigate safer alternatives to diclofenac, which may be equally efficacious in animals. Therefore, we chose:

1) to develop a chicken experimental model to study the toxicity profile of different NSAIDs because chickens are readily available, easily handled and have well studied physiological parameters.

2) to study the toxicity of diclofenac and other NSAIDs in the model, and

3) to compare the efficacy of safer alternative candidate NSAIDs in experimental and clinical situations.

Possible adverse effects of decline in vulture population

Environment

*Gyps* vultures are extremely effective and efficient scavengers. For example, in the Serengeti ecosystem in Tanzania, vultures are the major consumers of dead ungulates, accounting for greater meat consumption than all mammalian carnivores combined (Houston, 1979). A similar situation likely occurred in India prior to the declines, particularly in many regions where *Gyps* vultures were the primary scavengers.

Disease Spread

The effective loss of *Gyps* vultures from the Indian subcontinent is an important concern, both for the environment and for human health and well-being. For example, an abundance of unconsumed carcasses (Prakash *et al.* 2003) poses a direct threat to public health because the putrefying carcasses provide a good environment for potentially pathogenic bacteria, posing the possibility of direct or indirect infection. Unconsumed carcasses are also likely to provide sources of disease, such as anthrax, for humans, livestock, and wildlife. In removing carcasses of large ungulates rapidly and efficiently, vultures cleanse the environment. The
acidic conditions in the stomach of *Gyps* vultures kill many pathogenic bacteria, such as anthrax, reducing the risk of disease spread (Houston & Cooper 1975).

**Ecological imbalance**

The ecological extinction of *Gyps* vultures in India is also leading to further changes in the species complement of scavengers. The availability of surplus food, for example, appears to be driving a rapid and marked increase in the number of small, predatory mammals, such as feral dogs as well as rats (Cunningham *et al.*, 2001). Such species have relatively short lives and high reproductive potential. At one carcass dump in western Rajasthan, the numbers of dogs increased from approximately 60 in 1992 to >1200 in 2000 (Prakash *et al.* 2003). Because carcass dumps are often close to human habitation, they create a growing problem of dog attacks on people, which can be fatal. In addition, increasing populations of feral rats and dogs pose a significant risk of infectious disease to human beings, livestock, and wildlife. As the populations of feral rats and dogs increase, the rates of infectious disease transmission are likely to increase within these populations and from these species to others. Important zoonotic diseases, such as rabies and bubonic plague, which are endemic within India and for which dogs and rats, respectively, are the primary reservoirs, are likely to increase as a consequence of the vulture declines. More humans die from rabies in Asia than other regions, and the majority of these deaths occur in India (World Health Organization, 1998).

Wildlife and domestic livestock may also be at increased risk from dog- and rat-borne pathogens, including rabies, canine distemper virus, canine parvovirus, and *Leptospira* spp. bacteria. The increase in mammalian scavengers at carcasses may have unknown ecological consequences. Most mammalian scavengers are also predatory, and increases in their
populations as a result of the abundance of carrion are likely to lead to higher predation pressure on wildlife, such as mammals, ground-nesting birds, reptiles, and amphibians (Pain et al., 2002).

**Historical Research**

The first recognition of the recent decline in vulture populations in Southeast Asian region was observed in Keoladeo National Park, India. Affected birds were seen perched on the trees, dozing, with the neck slowly slumping down. The birds usually remained in this condition for more than thirty days before falling and death. About a 97% decline in vulture population was recorded (Prakash et al., 2003). A study conducted in the Punjab province of Pakistan showed a comparable decline in Gyps vulture population as observed in India. A total of 668 sick and dead vultures were collected of which 591 were less than one month postmortem. The minimum annual mortality rate in the adult breeding population was calculated to be 11.4 to 18.6%. Visceral gout in adults and sub-adults was found to be 63% and 80% respectively (Gilbert et al., 2002). In 2000, a survey was conducted to quantify the decline in the populations of *Gyps bengalensis* and *Gyps indicus* across India since 1990-1993. It was observed that a massive decline in the populations of both species were apparent from all parts of the country, and exceeded 92% overall. Apparently sick birds, with drooping necks, were observed in all regions and dead adult and juvenile vultures were frequently observed. The pattern of decline and the presence of sick and dead birds indicated epidemic disease as a possible cause (Prakashe et al., 2003).

**Previous declines in other avian species**

Declines in different bird species have been observed in the past, with attribution to a number of human-caused factors, including habitat destruction and contamination of food. However,
the use of the pesticide dichlorodiphenyltrichloroethane, or DDT, which affected the reproductive capabilities of these birds, was generally recognized as the most significant cause.

An unprecedented decline of peregrine falcon (*Falco peregrinus*) throughout much of the northern hemisphere was observed (Hickey, 1969). It was reported that broken eggs were much more common in 1949-56 than they had been in earlier years (Ratcliffe, 1958). Subsequently, Ratcliffe (1967) showed a significant decrease in the eggshell weight of peregrines in Britain starting in 1947 or 1948. Later, the same finding was demonstrated in North America (Hickey and Anderson, 1968). The relation of eggshell thickness to DDE residue levels was established for Alaskan peregrines by Cade *et al.*, (1971) and Peakall *et al.*, (1975). Peakall (1976) stated that pesticides were considered to be a major factor in the decline of peregrine and tentatively concluded that the levels of DDE in eggs that failed to hatch was 15-20ppm.

Populations of bald eagles and ospreys also underwent serious declines and organochlorine insecticides were implicated to be responsible (Mattson, 2000; Puleston, 1976). Patterns of reproductive failure in declining populations of several European and North American raptorial species were duplicated experimentally with captive American sparrow hawks (*Falco sparverius*) that were given a diet containing two commonly used organochlorine insecticides i.e. dieldrin and DDT. Major effects observed on reproduction, were increased egg disappearance, increased egg destruction by parent birds, and reduced eggshell thickness (Porter and Wiemeyer, 1969).

**Potential causes of rapid vulture population declines:**

Multiple reasons for this decline were hypothesized as mentioned below:
1. Loss of nesting habitat.
2. Exploitation and persecution.
3. Effects of transportation, infrastructure, and recreation.
4. Deliberate poisoning of carnivores leading to secondary poisoning of vultures.
5. Low food availability.
6. General environmental contamination.
7. Infectious diseases.
8. Use of veterinary drugs.

Numbers 1 to 4 above were not consistent with the observed steep decline in vulture populations and there is no supporting data for these assumptions, available in the literature.

Across the Indian sub-continent, there is considerable evidence that food availability for vultures has remained high. During nationwide vulture surveys in India in 2000, (Prakash et al., 2003) recorded numbers of livestock carcasses seen and any scavengers present. Only 12 (<5%) of 262 carcasses seen had attendant vultures, whereas most were attended by crows *Corvus* spp. and feral dogs. Counts of *Gyps* vultures at three carcass dumps that remained active between 1990 and 2000 showed 87-100% declines in the numbers of visiting vultures. In 1999, of 1,920 completed questionnaire returns, and around 80% of respondents indicated that dumping of carcasses in the open remained the predominant form of disposal in their region (Prakash et al. 2003). Whilst carcasses remained common and available to vultures, there was some indication that carcasses were less abundant than 10 years ago (76% of respondents reported carcasses as fairly or very common in 1990; 63% in 2000). Although few data exist, there is some evidence that the red-headed vulture underwent a significant (p=0.03) but less severe (48%) population decline between 1991-93 and 2000 (Prakash et al.
2003). This is further supported by a reanalysis of these data including the results for 2003 (Prakash et al. in prep). It is conceivable that, in the absence of the mortality factor that has caused the *Gyps* population crash, numbers of avian scavengers could be declining slowly in India due to a gradual reduction in available food. However, although monitoring data are scarce, populations of other scavenging birds show no obvious signs of decline, and some scavengers, such as feral dogs, are reported to be increasing across India (Cunningham et al., 2001). Finally, there has been no evidence of starvation being a contributing factor to the death of vultures necropsied from across India and Pakistan (Gilbert et al. 2002; Prakash et al. 2003). Consequently, food shortage is an unlikely explanation for the recent vulture population crash across the Indian sub-continent.

Postmortem examinations were carried out on 42 white-backed vultures from Pakistan, collected between 2000 and 2002 (33 adult and 9 juvenile birds). Of these, 28 birds had visceral gout, 14 did not. These birds were screened for a wide range of contaminants: cadmium (39), mercury (37), arsenic, copper, iron, manganese, molybdenum, zinc (all 39), carbamate and organophosphorous insecticides (34), organochlorine pesticides and polychlorinated biphenyls (13). Most tests were either negative or found concentrations below those consistent with a toxicosis. There was one case of lead toxicosis in a non-gout case and one case of probable organophosphorous insecticide poisoning. No deficiencies of essential elements were apparent (Oaks et al., 2004).

The most consistent postmortem finding in examined vulture carcasses was visceral gout, an accumulation of uric acid within tissues and on the surfaces of internal organs. Visceral gout is caused by renal failure, which is known to occur as a result of metabolic, infectious or toxic disease (Crespo and Shivaprasad, 2003). Visceral gout has been observed in
approximately 85% of dead adult and sub-adult birds collected in Pakistan (Oaks et al. 2004). In India, previous reports of lesions in vultures include both vultures captured prior to death and carcasses collected in the field. Of the small sample of carcasses collected in India, 75% of adult and sub-adult wild birds found dead had visceral gout (Cunningham et al. 2003). Other postmortem findings in examined birds both with and without visceral gout include enteritis, vasculitis, ganglioneuritis and gliosis (Cunningham et al. 2003). However, whilst the incidence of these lesions appeared to be high, the lesions themselves were generally subtle. Both the disruption of tissues by uric acid crystals and the presence of postmortem autolytic changes in birds found dead with gout would be expected to mask other lesions that may have been present. It is not possible, therefore, to know the true incidence of lesions such as vasculitis or gliosis in the birds found dead with gout. Sick vultures in India became increasingly weak over days or weeks before death and exhibited ‘head droop’ with increasing frequency as they became further incapacitated (Prakash, 1999). Oaks et al. (2004) failed to find evidence of avian influenza and West Nile virus, infectious diseases associated with renal failure, in Gyps bengalensis found dead in Pakistan. Attempts to isolate viruses from the kidney, spleen, lung and intestine of these birds were unsuccessful. Oaks et al. (2004) identified a novel mycoplasma by PCR in Gyps bengalensis found dead in Pakistan. The prevalence of this mycoplasma was similar in birds with and without visceral gout. Captive Gyps bengalensis were given a preparation made from tissues of vulture carcasses, including individuals with and without gout, to test for transmission of the mycoplasma or other infectious agent. No signs of disease occurred in the inoculated birds within 6 weeks of treatment. The results of some of the pathological studies on vultures from India suggested the presence of an infectious, probably viral, etiology. Visceral gout,
enteritis, vasculitis and gliosis is indicative of some infectious disease. However, the lesions are not clear to declare the infectious disease as a definite cause of huge mortality in vultures (Cunningham et al. 2003).

Later, Oaks et al. (2004) reported that 219 of 259 adult and sub-adult *Gyps bengalensis* found dead in Pakistan had visceral gout. In Pakistan, twenty-five *Gyps bengalensis* that were found dead with evidence of gout had detectable levels of the veterinary drug diclofenac in their kidneys, whereas diclofenac was not detectable (detection limit 0.005-0.01 mg kg-1) in any of 13 birds that did not have gout. Based on this perfect correlation between the incidence of gout and the presence of diclofenac and the high incidence of visceral gout in adult and sub-adult *Gyps bengalensis* found dead in Pakistan, it can be estimated that 85% of dead vultures of these age classes contained residues of diclofenac. Evidence suggests that the situation was broadly similar in India (Shultz et al. 2004).

Experimental treatment of captive *Gyps bengalensis* with diclofenac and tissues from livestock that had been treated with diclofenac showed that the birds were killed by consuming tissues of animals treated with the normal veterinary dose of diclofenac a few hours before slaughter (Oaks et al. 2004). The mortality rate of treated vultures was dose-dependent and indicated a median lethal dose of about 0.1 mg kg-1 (dose per unit vulture body weight). The experiment with captive birds also indicated that virtually all *Gyps bengalensis* consuming 0.8 mg kg-1 would be killed. Assuming that mortality rates of wild *Gyps bengalensis* are similar to those of captive birds and that a vulture’s average meal size is sufficient to supply 3 days’ free-living energy requirements, it would be expected that an average concentration of 0.5 mg kg-1 in ungulate tissue consumed by *Gyps bengalensis*
would be sufficient to deliver the median lethal dose and that 3.7 mg/kg would be sufficient to kill virtually all birds.

**Non-steroidal anti-inflammatory drugs (NSAIDs):**

The use of willow bark for the treatment of fever has been attributed to Hippocrates but clearly documented by Rev. Edmund Stone in a 1763. Leroux, and Pina crystallized salicin in 1829 from meadowsweet (*Spiraea ulmaria*), from which the name aspirin is derived. They isolated salicylic acid from this plant in 1836. Salicylic acid was acetylated by a French chemist, Gerhardt in 1853 and later on, Kolbe synthesized it in 1859 (Anne *et al*., 2005). Thus, acetylsalicylic acid, aspirin, became the first and prototype NSAID. The main therapeutic actions of aspirin are its effects as an antipyretic, anti-inflammatory, and analgesic drug. Over time, several other drugs were discovered that shared some or all of these actions, including antipyrine, phenacetin, acetaminophen (paracetamol), phenylbutazone, and more recently the fenamates, indomethacin, ibuprofen, and naproxen.

As a result of their similar therapeutic actions, these drugs tended to be regarded as a group and generally became known as the “aspirin-like drugs”; furthermore, as these drugs were clearly distinct from the glucocorticosteroids (the other major group of agents used to treat inflammation), they were also classed as the “non-steroidal anti-inflammatory drugs” (Flower, 1974).

NSAIDs belong to a class of chemically diverse compounds having similar mechanisms of action. Vane, Smith, and Willis reported that aspirin and NSAIDs act by inhibiting prostaglandin biosynthesis (Vane, 1971). The therapeutic, toxic, and anti-inflammatory properties of different NSAIDs are directly related to the amount and type of prostaglandin production that is impeded (Robinson, 1989).
In the USA, a prescription audit for 1999–2000, only 2 years after the introduction of COX-2 inhibitors onto the market, showed that there were 111 million prescriptions for NSAIDs, and one-third of these were for COX-2 agents. NSAIDs including aspirin also represented 60% of the total sales of over-the-counter (OTC) analgesics (Laine, 2001).

A homogeneous, enzymatically active cyclo-oxygenase (COX) or prostaglandin endoperoxide synthase was isolated by Hemler et al., (1976). This glycoprotein was shown to exhibit COX activity, including formation of cyclized arachidonic acid and addition the 15-hydroperoxy group to form prostaglandin G2. The hydroperoxy group of prostaglandin G2 is reduced to the hydroxyl group of prostaglandin H2 by a peroxidase that utilizes a wide variety of compounds to provide the requisite pair of electrons. Both COX and hydroperoxidase activities were contained in the same dimeric protein molecule (Smith, 1986).

We now know that COX exists in at least two distinct isoforms, COX-1 and COX-2 (Vane et al., 1998). Garavito and colleagues have determined the three-dimensional structure of COX-1, providing new understanding for the actions of COX inhibitors. This bi-functional enzyme comprises three independent folding units: an epidermal growth-factor-like domain, a membrane-binding motif, and an enzymatic domain. The sites for peroxidase and COX activity are adjacent but spatially distinct. The confirmation of a membrane-binding motif strongly suggests that the enzyme integrates into only a single leaflet of the lipid bi-layer and is thus a monotopic membrane protein. Three of the helices of the structure form the entrance to the COX channel, and their insertion into the membrane could allow arachidonic acid to gain access to the active site from the interior of the bi-layer (Picot et al., 1994).
Cyclooxygenase enzymes COX-1 or COX-2 catalyze the conversion of arachidonic acid to prostaglandin (PG) G₂ and H₂. Prostaglandin H₂ is converted into PGE₂, PGD₂, PGF₂α, PGI₂, and thromboxane, TX A₂ (DuBois et al., 1998) [Fig. 2.1].

Historically, COX-1 has been considered constitutive and mainly responsible for certain normal physiological functions (e.g., renal and gastrointestinal protection, blood clotting), and its inhibition has been the main cause of most NSAID-induced side effects such as gastric ulcers, renal failure, and blood dyscrasias (Lee, 2004b). The inducible isoform, COX-2, is induced in a number of cells by pro-inflammatory stimuli (Seibert et al., 1997; Tomlinson and Blikslager, 2003; Xie et al., 1992). Its existence was first suspected when Needleman and his group reported that bacterial lipopolysaccharide increased the synthesis of prostaglandins in human monocytes in vitro (Fu et al., 1990) and in mouse peritoneal macrophages in vivo (Masferrer et al., 1990). Sirois and Richards (1992) identified this inducible COX as a distinct isoform of COX (COX-2). The COX-2 enzyme is usually induced by inflammation, and its inhibition has been considered to be responsible for most therapeutic effects e.g., analgesic, anti-inflammatory effects (Lees, 2004b). The COX-1
sparing NSAIDs were developed for, and shown to have, a better gastrointestinal (GI) safety profile than the traditional, non-selective NSAIDs (Bombardier et al., 2000; Schnitzer et al., 2004). Maintenance of renal blood flow in the face of increased arterial tone is accomplished by the vasodilatory effects of prostaglandins (Wright, 2002).

Immunohistochemical studies have demonstrated effects of COX-1 on prostaglandin activity in many renal tissues, including the arterioles, collecting ducts, and glomeruli (Smith and Bell, 1978).

Surprisingly, the constitutive expression of COX-2 has recently been described in the cells of the macula densa. In the macula densa, the production of COX-2-related prostaglandins has been shown to increase in animals following salt and water restriction, thereby indicating that renal physiology may rely on the presence of both COX-1 and COX-2-related prostaglandins (Harris et al. 1994).

**Mechanism of action of NSAIDs**

All traditional NSAIDs (tNSAIDs) exert their action through inhibition of prostaglandin G/H synthase enzymes (COXs) and compete in a reversible manner with the arachidonic acid (AA) substrate at the active sites of COX-1 and COX-2 (Adams, 2001). Aspirin irreversibly inhibits the COX enzymes by acetylating serine residues in the active site and hence is often distinguished from other NSAIDs (Roth et al., 1975).

Selectivity for inhibitors is conferred by alternative conformations at the NSAID-binding site in the COX channel, such as a wider entrance and a secondary internal pocket (Luong et al., 1996). Selective COX-2 inhibitors comprise a subclass of NSAIDs that inhibits COX-2 while sparing COX-1. Acetaminophen has analgesic and anti-inflammatory action and possesses superior overall gastrointestinal safety profile compared with NSAIDs. Recently
defined cardiovascular warnings for use of COX-2 inhibitors should also be considered for acetaminophen in view its substantial COX-2 inhibition (Hinz et al., 2008).

COX-1 and COX-2 differ in their sensitivity to inhibition by certain anti-inflammatory drugs (Marnett et al., 1999).

The range of activities of NSAIDs against COX-1 compared with COX-2 largely explains observed variations in the side effects of NSAIDs at their anti-inflammatory doses. Drugs that have the highest potency against COX-2 and a more favorable COX-2 : COX-1 activity ratio will have potent anti-inflammatory activity with fewer side-effects on the stomach and kidney than agents with a less favorable COX-2 : COX-1 activity ratio. Aspirin, indomethacin, and ibuprofen are much less active against COX-2 than against COX-1 (Meade et al., 1993). Indeed, the most potent inhibitors of COX-1, such as aspirin, indomethacin, and piroxicam, are the NSAIDs that cause the most damage to the stomach (Lanza, 1989). The spectrum of activities of 10 standard NSAIDs against the two enzymes was shown to range from a high selectivity toward COX-1 (166-fold for aspirin) to equi-activity on both enzymes (Akarasereenont et al., 1994). Selective COX-2 inhibitors such as celecoxib, roficoxib have many fold greater potency against COX-2 than against COX-1 (Seibert et al., 1994; Chan et al., 1995). Other drugs which show COX-2 selectivity are meloxicam, nimesulide, and diclofenac (FitzGerald and Patrono, 2001; Laine, 2001; Rang et al., 1999; Warner et al., 1999) [Fig. 2.2].
Fig. 2.2: COX-1:COX-2 inhibition ratio of various non-steroidal anti-inflammatory drugs (FitzGerald and Patrono, 2001).
Based on their activity, NSAIDs can be classified as nonselective COX inhibitors and selective COX-2 inhibitors (Table-1) [Roberts II and Morrow, 2001].

Table-2.1: Chemical classification of analgesic, antipyretic and anti-inflammatory drugs.

<table>
<thead>
<tr>
<th>Nonselective COX Inhibitors</th>
<th>Selective COX-2 Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salicylic acid derivatives</em></td>
<td><em>Para-aminophenol derivatives</em></td>
</tr>
<tr>
<td>Aspirin, sodium salicylate, choline magnesium trisalicylate, salsalate, diflunisal, sulfasalazine, olsalazine</td>
<td>Acetaminophen</td>
</tr>
<tr>
<td><em>Indole and indene acetic acids</em></td>
<td><em>Diaryl-substituted furanones</em></td>
</tr>
<tr>
<td>Indomethacin, sulindac</td>
<td>Rofecoxib</td>
</tr>
<tr>
<td><em>Heteroaryl acetic acids</em></td>
<td><em>Diaryl-substituted purazoles</em></td>
</tr>
<tr>
<td>Tolmetin, diclofenac, ketarolic</td>
<td>Celecoxib</td>
</tr>
<tr>
<td><em>Arylpropionic acids</em></td>
<td><em>Indole acetic acids</em></td>
</tr>
<tr>
<td>Ibuprofen, naproxen, flurbiprofen, ketoprofen, fenoprofen, oxaprofen</td>
<td>Etodolac</td>
</tr>
<tr>
<td><em>Anthranilic acids (fenamtes)</em></td>
<td><em>Sulfonanilides</em></td>
</tr>
<tr>
<td>Mefenamic acid, meclofenamic acid</td>
<td>Nimesulide</td>
</tr>
<tr>
<td><em>Enolic acids</em></td>
<td></td>
</tr>
<tr>
<td>Oxicams (piroxicam, meloxicam)</td>
<td></td>
</tr>
<tr>
<td><em>Nicotinic acid derivatives</em></td>
<td></td>
</tr>
<tr>
<td>Flunixin</td>
<td></td>
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<tr>
<td><em>Alkanones</em></td>
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<tr>
<td>Nabumetone</td>
<td></td>
</tr>
</tbody>
</table>

Inflammation is a group of responses to a variety of noxious stimuli including infections, antibodies and physical injuries. It can be essential for survival in the face of environmental
pathogens and injury. However, in some situations and diseases, the inflammatory response may be exaggerated and sustained without apparent benefit, and even with severe adverse consequences. Several mechanisms have been proposed to explain the inflammatory process (Kyriakis and Avruch, 2001; Serhan and Chiang, 2004). Earlier studies indicated the promotion of cellular migration out of the microvasculature as the possible mechanism of development of inflammation but recent work has focused on adhesive interactions, including the E-, P-, and L-selectins, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and leukocyte integrins, in the adhesion of leukocytes and platelets to endothelium at sites of inflammation (Meager, 1999). Some traditional NSAIDs may interfere with adhesion by inhibiting expression or activity of certain of these cell-adhesion molecules (Diaz-Gonzalez and Sanchez-Madrid, 1998). Some mediators including complement factor C5a, platelet-activating factor, and the eicosanoid, leukotriene B4 (LTB4), attract inflammatory cells to the site of injury. Several cytokines, especially interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) secreted by a variety of cells, mediate inflammatory process (Dempsey et al., 2003). All aforementioned mediators exert their effects probably, by enhancing the production of prostaglandins. All anti-inflammatory drugs inhibit prostaglandin synthesis, and hence reduce inflammation. Histamine, bradykinin and serotonin are other inflammatory mediators, but they are not inhibited by NSAIDs. The anti-inflammatory effect of NSAIDs is primarily, the consequence of inhibition of COX-2 enzyme by these compounds. At higher concentrations, NSAIDs also are known to reduce production of superoxide radicals, induce apoptosis, inhibit expression of adhesion molecules, decrease nitric oxide synthase, decrease pro-inflammatory cytokines (e.g., TNF-α, interleukin-1), modify lymphocyte activity, and alter cellular membrane functions. However,
there are differing opinions as to whether these actions might contribute to the anti-inflammatory activity of NSAIDs (Vane and Botting, 1998) at the concentrations attained during clinical dosing in people. The hypothesis that their anti-inflammatory actions in humans derive from COX inhibition alone has not been rejected based on current evidence.

Pain accompanying inflammation and tissue injury results from local stimulation of pain fibers and enhanced pain sensitivity (hyperalgesia) is, in part, a consequence of increased excitability of central neurons in the spinal cord. The capacity of prostaglandins to sensitize pain receptors to mechanical and chemical stimulation apparently results from a lowering of the threshold of the polymodal nociceptors of C fibers. NSAIDs do not affect either hyperalgesia or pain caused by the direct action of prostaglandins, however, some data have suggested that relief of pain by these compounds may occur via mechanisms other than inhibition of prostaglandin synthesis, including antinociceptive effects at peripheral or central neurons (Gebhart and McCormack, 1994; Konttinen et al., 1994). The role of COX enzyme in mediation of the perception of pain is not clear.

Fever may be the response of infection, tissue damage, inflammation, graft rejection or malignancy. These situations stimulate the production of cytokines such as IL-1α, IL-6, interferons, and TNF-α, which enhance PGE₂ synthesis in and around the hypothalamic area. PGE₂, in turn, increases cyclic AMP and triggers the hypothalamus to elevate body temperature by promoting an increase in heat generation and a decrease in heat loss. It is proposed that NSAIDs inhibit the synthesis of PGE and promote body temperature return to normal (Dascombe, 1985).
Moses et al. (2001) observed that phenylbutazone, flunixin meglumine, ketoprofen, carprofen, meloxicam, and a low-concentration of methyl-prednisolone suppressed PGE2 production in LPS-challenged explants in equine synovial membrane. Meloxicam appeared to have more selective suppression of COX-2 activity.

**Therapeutic applications of NSAIDs in human beings**

NSAIDs find their chief clinical application as anti-inflammatory agents in the treatment of musculoskeletal disorders, such as rheumatoid arthritis, osteoarthritis, ankylosing spondylitis and gout. In general, NSAIDs provide only symptomatic relief from pain and inflammation associated with the disease, do not arrest the progression of pathological injury to tissue, and are not considered to be "disease-modifying" anti-rheumatic drugs (Kean and Buchanan, 2005; Schnitzer, 2003; Simon, 1997; Zochling et al., 2006).

It is proposed that NSAIDs are suitable to control the pain of low to moderate intensity such as postoperative dental pain (Ong and Seymour, 2003) and headache in humans (Lipton et al., 1998). Prostaglandins released from endometrium during menstruation are responsible for the symptoms of dysmenorrhea. NSAIDs have a good reputation for the treatment of this condition (Connolly, 2003; Marjoribanks et al., 2003; Morrison et al., 1999).

It is observed that COX-2-mediated prostaglandins predominantly are involved in elevation of body temperature evoked by bacterial lipopolysaccharide (LPS), hence selective COX-2 inhibitors may be superior than tNSAIDs as antipyretic agents (McAdam et al., 1999).

Although it is reported that both COX-1 and COX-2 participate in the maintenance of patency of the ductus arteriosus in the fetal lamb (Clyman et al., 1999), and that only COX-2 is responsible in mice (Loftin et al., 2002), it is not clear which isoform(s) is involved in
maintaining this situation in humans. In many cases, indomethacin and other tNSAIDs are used to treat this condition in neonates.

NSAIDs, especially aspirin and ketoprofen, are used to treat mastocytosis, in which PGD₂ released from mast cells cause vasodilation and hypotension in patients (Worobec, 2000). Hypocalemic, hypochloremic metabolic alkalosis with normal blood pressure and hyperplasia of juxtaglomerular apparatus are the characteristics of Bartter’s syndrome. Renal COX-2 is induced and biosynthesis of PGE₂ is increased in this syndrome. Selective COX-2 inhibitors also have been used to improve biochemical derangements and the symptoms (Guay-Woodford, 1998). This type of syndrome is effectively treated with indomethacin in infants (Seyberth et al., 1987).

Another potential area for the pharmacological application of NSAIDs is in cancer chemoprevention. Use of aspirin is reported to be significantly effective in reducing the risk of colon cancer and some other types of cancer in human beings (Kune et al., 1998; Jacobs et al., 2004). Other NSAIDs such as sulindac, celecoxib, and rofecoxib have also been shown efficacious in reducing polyp recurrence in familial adenomatous polyposis (Cruz-Correa et al., 2002; Hallak et al., 2003; Steinbach et al., 2000).

Niacin is clinically used to lower serum cholesterol levels but is often not well tolerated due to its ability to cause flushing via release of prostaglandin D (PGD) from skin. Aspirin is used successfully to overcome this vasodilatory response (Jungnickel et al., 1997).

Diclofenac is a non-steroidal anti-inflammatory drug that is widely prescribed for the treatment of osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, acute musculoskeletal injury, postoperative pain, and dysmenorrhea (Brogden et al, 1980; Small, 1989).
Therapeutic uses of NSAIDs in animals

NSAIDs are commonly used in veterinary medicine for the treatment of inflammation of musculoskeletal and other tissues (e.g. spondylitis, laminitis, mastitis), endotoxic shock and colic and for the control of pain, associated with trauma or surgery (Boothe, 2001). These drugs may favorably influence the course and outcome of these and other diseases and disorders. The ability of non-steroidal anti-inflammatory drugs to suppress inflammation and subsequent tissue damage is important, since the inflammatory process may result in organ damage that renders the animal unprofitable or useless for production (Kopcha and Alwynelle, 1989). Also the suppression of pain, which causes distress to the animal, is an important pharmacological benefit of NSAIDs (Danbury et al., 1997). NSAIDs are employed in animals for their analgesic, antipyretic and anti-inflammatory activities. These compounds are prescribed to control pain of varying nature such as trauma, postoperative situations, osteoarthritis, dental diseases, and cancer in critically ill animals (Bernard, 2005).

In ruminants, NSAIDs are used to treat various clinical conditions such as arthritis, spondylitis, and laminitis (Williams, 1988). These drugs are also advantageous in treatment of endotoxic mastitis (Anderson, 1986a, b; Jarlo, 1992; Dascanio et al., 1995; Wagner and Apley, 2004), metritis (Amiridis et al., 2001) and bovine respiratory disease (Balmer et al., 1997; Bureau et al., 1998; Lockwood et al., 2003). Endotoxemia in neonatal calves is also treated with NSAIDs (Semrad, 1993). There is also a growing trend of using these drugs in certain surgical procedures such as dehorning and castration to minimize pain (Sutherland et al., 2002; Ting et al., 2003).

NSAIDs are good therapeutic agents to treat lameness due to laminitis, navicular disease, osteoarthritis and other musculoskeletal disorders, colic and endotoxemia in horses (Owens
et al., 1996; Raekallio et al., 1997; Hardie et al., 1985; Lee and Higgins, 1985; Tobin, 1979). In pigs, NSAIDs are indicated against endometritis-agalactia syndrome, fever and pain (Hirsch et al., 2003). Dogs and cats are treated with NSAIDs for a variety of clinical conditions including osteoarthritis, postoperative pain, septic shock and neoplasia (Davidson et al., 1992; Knapp et al., 1992; Möllenhoff et al., 2005; Slingsby and Waterman-Pearson, 2000; Welsh et al., 1997).

Various indications in birds exist for which NSAIDs could be potentially beneficial. Their use in trauma and surgery may be helpful in controlling pain. These compounds are indicated for respiratory diseases, digestive and coccidial infections to sustain good weight gain (Cristófol et al., 2000). Indomethacin, nimesulide and ibuprofen have shown some efficacy against intestinal coccidiosis (Hornok et al., 1999; Allen, 2000; Vermeulen, 2002). Broiler ascites may be the result of multiple factors including genetics, metabolic and environmental (Decuypere et al., 2000; Julian, 2000). Although the mechanism of effect is not clear, treatment with aspirin was found to be beneficial in this syndrome (Balog et al., 2000).

Variable effects of NSAIDs in laying hens and broiler chickens, including slight improvement in egg production, egg shell quality and survival rates during heat stress have been reported (Abd-Ellah et al., 1997; Balog and Hester, 1991; Oliver and Birrenkott, 1981). NSAIDs are employed in alleviating lameness in chickens, turkeys and ducks (Jouglar and Benard, 1992; Hocking et al, 1997; McGeown et al., 1999). Beak trimming in chicken is associated with acute pain and reduced feed intake (Hughes and Gentle, 1995). This condition may be another indication for NSAID therapy.
Adverse effects of NSAIDs in human beings

NSAIDs exert their activity mainly via inhibition of prostaglandins by interacting with COX enzymes. The adverse effects of these drugs are also mediated through the impairment of COX enzymes and thus prostaglandins. It is generally accepted that COX-1 inhibition is responsible for the majority of these effects but there is adequate evidence that inhibition of COX-2 also contributes toward the toxicity of NSAIDs (Mizuno et al., 1997). It is supposed that COX-1 enzyme is constitutively expressed in tissues and mediates the synthesis of beneficial prostaglandins while COX-2 is inducible in the response of inflammatory stimuli (Lee et al., 2004b). However, recent studies indicate that the latter isoenzyme is constitutively present, at least in gastric mucosa, kidneys and brain.

Cytoprotective prostaglandins, especially, PGI₂ and PGE₂, synthesized by COX-1 inhibit gastric acid, enhance mucosal blood flow, and promote mucus and bicarbonate secretions in stomach and intestine. Studies in animals suggest that inhibition of COX-1 and COX-2 is required for induction of gastric ulcerogenic action of nonselective NSAIDs (ns-NSAIDs) (Wallace et al., 2000; Tanaka et al., 2001; Tanaka et al., 2002a, b; Takeuchi et al., 2004). Inhibition of these PGs by NSAIDs renders these organs susceptible to damage. These drugs also cause direct irritation to the gastric epithelium, which results in back diffusion of acid. Moreover these drugs stay in non-ionized form due to acidic medium of gastric lumen and get absorbed across the plasma membrane of epithelial cells and are subsequently ionized, resulting in intracellular trapping of H⁺ ions (Schoen and Vender, 1989). Implications to humans have included anorexia, nausea, abdominal pain, diarrhea, non-ulcer dyspepsia, and serious GI tract-related side effects, such as gastric and duodenal ulcers, erosions, bleeding, perforation, esophagitis, and esophageal strictures (Lanza et al., 1983; Bjorkman, 1996;
Mason, 1999; Schoenfeld et al., 1999; Scheiman, 2003). NSAIDs can also cause topical mucosal damage by diminishing the hydrophobicity of gastric mucus, thereby allowing endogenous gastric acid and pepsin to injure the surface epithelium (Wolfe and Soll, 1988). These drugs mostly cause anorexia, nausea, dyspepsia, abdominal pain and diarrhea. In some patients ulceration may range from superficial erosion to full thickness perforation. These ulcers may be single or multiple and can be accompanied by gradual blood loss leading to anemia or by life-threatening hemorrhage. Selective COX-2 inhibitors such as celecoxib, valdecoxib, rofecoxib and lemeracoxib may pose fewer gastrointestinal hazards (Deeks et al., 2002; Schnitzer et al., 2004).

Nonselective NSAIDs prevent the synthesis of platelet thromboxane A\textsubscript{2} (TXA\textsubscript{2}), a potent aggregating agent, hence increase the bleeding time. Aspirin is well known to possess this property because it irreversibly inhibits the COX in platelets. This side effect has been exploited in the prophylactic treatment of thromboembolic disorders. Naproxen may exhibit the similar activity (Capone et al., 2004) while other nonselective NSAIDs may not afford adequate cardioprotection from thromboemboli due to their shorter duration of action (Garcia Rodriguez et al., 2004). Selective COX-2 inhibitors suppress the platelet aggregation by TXA\textsubscript{2}, a possible mechanism of increased risks of thrombosis from use of selective COX-2 inhibitors (McAdam et al., 1999; Catella-Lawson et al., 2001). The incidence of myocardial infarction and stroke in patients at risk with selective COX-2 were compared to nonselective NSAIDs (FitzGerald, 2003). Clinical studies revealed a higher incidence of these problems with rofecoxib (Bresalier et al., 2005), valdecoxib (Nussmeier et al., 2005), and celecoxib (Solomon et al., 2005) consistent with a mechanism-based cardiovascular hazard for the class (FitzGerald, 2003).
The levels of PGE$_2$ and PGF$_{2\alpha}$ increase markedly in response to the induction of COX-2 in myometrium and have important role during labor (Slater et al., 2002). Use of NSAIDs near term may increase the risk of closure of ductus arteriosus, delayed parturition and postpartum hemorrhage.

Renal and renovascular adverse events are associated with the use of NSAIDs (Cheng and Harris, 2004). These effects include acute tubular necrosis, acute tubulointerstitial nephritis, glomerulonephritis, renal papillary necrosis, chronic renal failure, salt and water retention, hypertension, hyperkalaemia and hyperreninaemic hypoaldosteronism (Tse and Adu, 1998). There are reports of sub-clinical renal dysfunction due to NSAIDs (Calvo-Alen et al., 1994). NSAID-associated adverse renal effects are attributed to the inhibition of prostaglandins, which are responsible for maintaining renal blood flow and the glomerular filtration rate, especially in fluid depleted individuals. This permits unopposed vasoconstrictive action of leukotrienes, angiotensin II, vasopressin, endothelin and catecholamines. In normal salt and water replete subjects, these do not result in reduction of glomerular filtration rate (GFR), but in states of renal hypoperfusion, unopposed glomerular vasoconstriction can result in acute renal failure (ARF). Additionally, NSAID-induced hyporeninaemia and hypoaldosteronism, along with the decreased distal tubular flow and sodium delivery, result in hyperkalaemia (Ejaz et al., 2004). Selective COX-2 inhibitors also have the propensity to cause such effects (Brater, 1999).

Diclofenac produces adverse drug reactions in about 20% of patients. These are mostly gastrointestinal effects, but depression of renal function and elevation of hepatic aminotransferases can also occur (O’Brien, 1986).
The effects of the non-steroidal anti-inflammatory drug diclofenac and the pyrazolone derivative dipyrone on renal function were compared with those of placebo in 12 healthy male human volunteers (Farker et al. 1995). They found that dipyrone and diclofenac at therapeutic dosages over 3 days were shown not to decrease glomerular filtration and renal plasma flow in healthy individuals.

Caspi et al. (2000) studied the effect of mini-dose aspirin (75 mg/day) on renal function and uric acid handling in 49 elderly patients (ages 61-94 years). They found that creatinine and uric acid clearance rates paralleled each other during aspirin treatment. However, one week after aspirin discontinuation, creatinine clearance remained decreased while uric acid clearance returned to baseline. Thus some amount of renal dysfunction seems to persist even though creatinine returns to normal. Koseki et al. (2001) studied 235 early rheumatoid arthritis patients who were being given NSAIDs. A rise in creatinine was found in 14 patients (6%). This was attributed to inhibition of prostaglandin synthesis due to NSAIDs in three cases. Serum creatinine improved on stopping the drug in all three cases.

Some patients may experience hypersensitivity to aspirin and other NSAIDs showing symptoms that range from vasomotor rhinitis with profuse watery secretions, angioedema, generalized urticaria, and bronchial asthma to laryngeal edema, bronchoconstriction, flushing, hypotension, and shock (Munir et al., 2007). Treatment with diclofenac in human beings has been associated with a rare, but severe, incidence of hepatic injury, which is often described as idiosyncratic toxicity (Banks et al., 1995; Boelsterli, 2003).

In human beings, tubular lesions in the kidneys are frequently observed with the use of NSAIDs, resulting in acute or chronic renal failure, mainly due to tubular necrosis (McCrary et al., 2002). A study by Garcia et al. (1997) showed that the use of NSAIDs is associated
with a high prevalence of gastrointestinal toxicity in humans. Wide variations in relative risk among NSAIDs were observed with piroxicam and azapropazone being the most toxic. Ibuprofen was associated with the least risk, probably because of its widespread use as a low-dose analgesic. Brunner et al., (1985) reported a case of a man with severe, reversible, non-oliguric renal failure caused by biopsy-proven acute interstitial nephritis after therapy with mefenamic acid (Ponstan).

**Human NSAID toxicity and concerns regarding tissues in food animals:**

The US Food and Drug Administration (FDA)banned the use of phenylbutazone in adult dairy cattle due to its adverse effects like fatal hypersensitivity reactions and blood dyscrasias in humans (FDA, 2003). Similarly, dipyrone, which was being used in food animal species illegally. The drug has been associated with serious toxic effects in humans. To protect humans from dipyrone residues, FDA now prevents its use in food animals (Payne et al., 1999).

**Manifestations of NSAID toxicosis in non-human animals:**

Like humans, animals experience adverse effects of NSAID therapy such as gastrointestinal lesions, nephrotoxicity and hematopoietic disturbances.

Erdem and Guzeloglu (2009) investigated the effects of meloxicam treatment during early pregnancy in Holstein heifers. They found that administration of meloxicam at the time associated with pregnancy recognition processes to maintain the corpus luteum decreased the pregnancy rate. Richy et al (2009) reported that piroxicam was globally safer than other NSAIDs notably indomethacin, naproxen and salicylates. From a global GI safety point of view, piroxicam was better tolerated than indomethacin, naproxen and salicylates while less
tolerated when compared to meloxicam. Major GI effects were comparable among piroxicam users as in comparator drugs users, except for meloxicam.

Swinkels et al., (1994) studied the adverse effects of the non-steroidal anti-inflammatory drugs (NSAIDs) ketoprofen (3 mg/kg) and flunixin (2 mg/kg) in pigs after endobronchial challenge with *Actinobacillus pleuropneumoniae*. At postmortem no adverse gastric and renal effects were observed for either drug.

Taylor et al., (1994) reported that six healthy adult female cats were given 1.0 mg/kg flunixin meglumine orally daily for seven consecutive days. No abnormal clinical signs were seen and appetite was unaffected throughout the study. Of course the domestic cat has a very low capacity to conjugate paracetamol (acetaminophen) because of its low glucuronyl transferase activity. Hence, cats are extremely sensitive to the toxic effects of paracetamol, and what is a therapeutic dose in other species may prove to be a lethal dose in the cat (Campbell & Chapman, 2000a). Cats are also more susceptible to the toxic effects of permethrin because, unlike some other mammalian species, they lack the necessary detoxification pathways (Martin & Campbell, 2000).

Although dogs can benefit from NSAID therapy for a variety of conditions, Fox and Johnston (1997) stated that canines are especially sensitive to several NSAIDs, and reports of serious, and occasionally fatal, complications are numerous. Dogs appear to be more sensitive to the effects of non-steroidal anti-inflammatory drugs (NSAIDs) on the gastrointestinal tract than many other species (Campbell & Chapman, 2000a). Chronic use of nonselective NSAIDs in dogs has been associated with serious GI tract side effects, manifested as bleeding, ulceration, erosions, perforations, peritonitis, melena, anemia, anorexia, and abdominal pain (Ricketts et al., 1998; Reed, 2002). Although there are reports
on the safety of selective COX-2 inhibitors in people, their safety has not been established in animals. In three clinically ill dogs, signs of gastroduodenal ulceration were first noticed within seven days of flunixin meglumine administration, and they included pyrexia, anorexia, weight loss, vomiting, melena, pain on abdominal palpation, and abdominal distention (Vonderhaar and Salisbury 1993). GI tract perforation was observed in dogs that received deracoxib, a COX-2 inhibitor, at a higher than approved dosage or when the animals had received at least one other nonselective NSAID in close temporal association with deracoxib administration (Lascelles et al., 2005). Knapp et al., (1992) reported that, a non-steroidal anti-inflammatory drug, was given to 62 dogs bearing naturally occurring tumors in a phase I clinical trial. Dose-limiting gastrointestinal irritation/ulceration occurred in dogs treated with piroxicam (1.5 mg/kg q48h) Subclinical renal papillary necrosis occurred in two of the dogs. Kietzmann et al., (1996) studied tolerance and pharmacokinetics of a combination of phenylbutazone and prednisolone (tablets) in Beagle dogs. No signs of intolerance were found. The glandular portion of the stomach was most severely affected by phenylbutazone, flunixin meglumine, and ketoprofen. In the phenylbutazone-treated group, but not in the other groups, edema of the small intestine and erosions and ulcers of the large colon were observed. The non-steroidal inflammatory drugs, meclofenamic acid and phenylbutazone, are associated with the induction of aplastic anaemia in dogs (Weiss & Klausner, 1990). Anaemia, thrombocytopenia and pancytopenia have also been reported in dogs following phenylbutazone treatments (Watson et al., 1980). Gastric-duodenal erosion and/or hemorrhage, and gastric ulceration were associated with phenylbutazone and flunixin administration in dogs and horses, respectively (Forsyth et al., 1998).
NSAIDs have the potential to cause acute renal failure in horses (Geor, 2003; Schmitz, 1988; Bayly, 2004). These drugs are used in the treatment of osteoarthritis and may have detrimental effects on proteoglycan (Beluche et al., 2001). Renal papillary necrosis has been reported in horse with the use of NSAIDs. In a retrospective study of 269 horses that had been treated with phenylbutazone, various signs and lesions were observed. Anorexia, depression, colic, hypoproteinemia, diarrhea, melena, weight loss, ventral edema, petechial hemorrhages of mucous membranes, oral and gastrointestinal tract erosions and ulcers, renal papillary necrosis, and death were among the complications seen in horses that had received greater than 8.8 mg/kg of body weight/day (Collins and Tyler, 1984). Also, oral ulceration has been observed in horses treated with phenylbutazone (Tobin et al, 1986). When a double-blind study was performed with horses to determine the potential toxic effects of the non-steroidal anti-inflammatory drug, eltenac few glandular gastric ulcers, mild in severity, developed in some of the animals during the treatment period (Goodrich et al, 1998). This occurred more often in horses treated with eltenac at doses of 0.5 mg/kg, 1.5 mg/kg and 2.5 mg/kg once daily, intravenously. Gross postmortem and histological examination did not reveal any signs of drug related gastrointestinal, renal or hepatic abnormalities. Toxic effects of eltenac given intravenously were greatest in horses treated with 2.5 mg/kg (highest dose). The relative toxicity of phenylbutazone, flunixin meglumine, and ketoprofen was studied in healthy adult horses. At necropsy, necrosis of the glandular portion of the stomach was observed in all treatment groups. In the phenylbutazone-treated group, edema of the small intestine and erosions and ulcers of the large colon were observed. Flunixin and phenylbutazone also caused renal crest necrosis. Renal lesions did not develop in ketoprofen-treated groups (MacAllister et al, 1993). Flunixin is reported to have the potential for
myonecrosis at injection site, hence, intravenous administration may be preferred (Pyorala et al., 1999).

Varela et al., (1998) reported that in Portugal, piroxicam is one of the most prescribed non-steroidal anti-inflammatory agents (NSAIDs), and usage is frequently associated to photosensitivity skin reactions in human. Karakaya and Kalyoncu (2002) reported that in humans the most common adverse reactions after oral administration of metamizole (dipyrone) were bronchospasm and urticaria in Turkey. Gomez et al., (2003) reported that diclofenac triggers apoptosis cascade in rat hepatocytes. It may be concluded that hepatotoxicity is one of the side effects associated with the administration of diclofenac.

Ford and Houston (1995) observed that for the same dose of diclofenac in rats caused variable intestinal damage when administered by subcutaneous bolus and intravenous infusion. The latter route of administration was associated with more severe lesions.

Birds are among the species that are prone to develop visceral and renal gout in concert with renal failure, dehydration, and other disorders. In birds, the clinical signs of kidney disease are similar to those shown by mammals, which become lethargic, weak and anorexic; regurgitation may occur (Speer, 1997).

Gout occurs if plasma sodium urate concentration exceeds its solubility. Visceral gout is the deposition of urate crystals on the visceral membranes, mostly on the pericardium, liver, and spleen. Urate deposits are also visible within the kidney. In acute renal failure visceral gout might occur alone (Lumeij, 1994). Inflammatory reactions are often not detected, as birds die rapidly. Visceral gout usually develops without obvious clinical signs prior to death. In cases where renal urate deposition occurs prior to visceral gout, anorexia and lethargy may be
noted for hours or days. Reptiles are also quite prone to visceral and renal gout. For example, Montali *et al.* (1979) observed visceral gout in snakes treated with gentamicin.

Gout in birds is more of a lesion consistent with renal failure than a distinct disease. The solubility is estimated as approximately 600 mol/L as a minimum level. As uric acid is excreted via tubular secretion 70% of the kidney must be malfunctioning to cause a hyperuricaemia in birds (Lumeij, 1994). Apart from renal failure, dietary protein above the bird requirements might also cause a hyperuricemia. Hyperuricemia can result in visceral or articular gout. In cases of renal failure without precipitation in the renal tubules, articular gout, or less commonly, visceral gout, occurs alone. Visceral gout is the deposition of urate crystals on the visceral membranes, mostly on pericardium, liver, and spleen. Urate deposits are also visible within the kidney. In acute renal failure, visceral gout might occur alone. In cases where renal urate deposition occurs prior to visceral gout, anorexia and lethargy may be noted for hours or days. Hyperkalaemia can develop, and this, rather than the uric acid, might lead to cardiac arrest and the sudden death seen with visceral gout. In postmortem examinations pericardium and liver membranes are white, resembling powdered sugar (Lumeij, 1994).

There are various causes of visceral gout in birds, including ochratoxicosis (Peckham *et al.*, 1971). Waterfowl inoculated with influenza viruses died and had lesions of visceral gout (Slemons *et al.*, 1990). Avian nephritis virus inoculation results in visceral gout in chickens (Shirai *et al.*, 1989). Urinary tract cryptosporidiosis was reported as the cause of visceral gout in commercial laying hens (Trampel *et al.*, 2000). Also oosporein and a mycotoxin were responsible for development of gout in broiler chickens and turkey poult's (Pegram and Wyatt, 1981; Pegram *et al.*, 1982). Excess sodium bicarbonate in the diet of layer and broiler
chickens results in visceral gout (Davison and Wideman, 1992; Ejaz et al., 2005). Mubarak and Sharkawy (1999) concluded that development of gout in birds may be related to a state of metabolic alkalosis which is associated with significant changes in electrolyte balance. Serum uric acid increased in captive sandhill cranes (*Grus canadensis*) after adding excess sea salt in drinking water to make sodium chloride concentration 1% (Franson et al., 1981).

The administration of 5·5 mg/kg flunixin meglumine (Banamine; Schering-Plough), 2·5 mg/kg ketoprofen (Ketofen; Merial) or 0·1 mg/kg meloxicam (Bioflac; Cristalia), for either three or seven days to budgerigars did not cause the typical clinical signs associated with renal disease or changes in the colour and consistency of their feces (Pereira and Werther, 2007).

Vultures of two species, *Gyps africanus* and *Gyps fulvus* expressed the clinical signs of lethargy and neck drooping and death after treatment with diclofenac 0.8 mg/kg. Extensive visceral gout was evident on postmortem examination of these birds (Swan et al., 2006a).

No renal impairments were observed in Japanese quails (*Coturnix coturnix japonica*) treated with 3·4 mg/kg flunixin meglumine for three days or 1·0 mg/kg meloxicam for five days (Lucas, 2003).

Indomethacin (2.5 mg/kg) causes a rapid increase in plasma urate concentrations in hens and at 5 mg/kg I.M. may cause death by deposition of urates in the viscera (visceral gout). This increase is probably a consequence of decreased renal tubular urate secretion. The sensitivity to indomethacin varies either according to the breed of bird or the physiological status and hens appear more sensitive during egg formation (Nys and Rzasa, 1983).

Signs of diclofenac toxicosis in domestic fowl given 10 mg/kg body weight at 24 h post-dosing, range from severe depression to death. These manifestations corresponded to
increases in plasma uric acid concentration (Naidoo et al, 2007). Postmortem examinations showed signs of gout with deposits of urates (tophi) in the kidneys, liver, heart and spleen. Pharmacokinetic studies with either intramuscular or oral administration showed that diclofenac had a short half-life of elimination of approximately 1 h, a volume of distribution of 0.09–0.24 l/kg and relative oral bioavailability of 50% compared to intramuscular administration.

Mishra et al. (2002) found visceral gout at postmortem in dead vultures during the large mortality event on the Indian subcontinent.

**Histopathology**

The comparative toxic effects of nimesulide and diclofenac were evaluated by Reddy et al. (2006) in Vanaraja and PB1 poultry breeds. The kidney sections of the birds treated with diclofenac sodium at the dosage rate of 5 mg/kg showed mild tubular degeneration with lymphoid aggregates, disruption of tubular architecture with mild inter-tubular fibrosis and marked inter-tubular congestion. In liver of the diclofenac-treated birds, severe sinusoidal and central vein congestion and bile duct hyperplasia were observed. However, other organs including heart, spleen and lungs did not show any gross or microscopic lesions.

Naidoo et al (2007) studied diclofenac toxicosis in 18-week-old Leghorn fowl. They were treated at various doses (0.6, 1.25, 2.5, 5 and 10 mg/kg). Renal histopathology revealed marked disruption of cortical architecture due to the presence of tophi containing both spiculate as well as globoid urate forms with both cell necrosis and tubule loss. Massive heterophil infiltration was present in the interstitium of the cortex, tubules and collecting ducts. Renal necrosis was most severe in the birds of the 10 mg/kg group that died. It was characterized by massive urate crystal precipitation with necrosis of the adjacent structures. It
was observed in most cases that, although the basement membrane of the tubules were still apparent, most of the tubular structure was lost due to the presence of large aggregates of urates. Rarely, the tophi had multinucleate giant cells at their periphery. There was infiltration of macrophages and lymphocytes in some areas of the interstitium and possible early fibroblast proliferation. The liver tissues in the birds given higher doses of diclofenac showed small tophi associated with hepatocyte necrosis. Mostly, the tophi were associated with an inflammatory reaction of heterophils and round cells. In some cases the tophi consisted of central aggregates of urate spicules with peripheral infiltration of fewer inflammatory cells.

Quails treated with different dosages of flunixin meglumine (0·1 to 32 mg/kg) developed glomerular lesions. The severity of the lesions and the presence of basophilic mineral deposits in the glomeruli, which were amorphous to granular, were related to the administered doses (Klein et al., 1994).

A low frequency of glomerular congestion was observed in budgerigars (Melopsittacus undulatus) treated with flunixin meglumine, ketoprofen and meloxicam for three days. The increase in mesangial matrix synthesis, which was usually related to the deposition of immunocomplexes, was observed only in birds treated with flunixin meglumine for three or seven days. At 5·5 mg/kg, flunixin meglumine caused increased glomerular mesangial matrix synthesis after three and seven days of treatment, and tubular necrosis in six of eight (75%) of the birds after seven days (Pereira and Werther, 2007).

Acute necrosis of the proximal convoluted tubules in vultures with visceral gout thought to have died as a consequence of diclofenac toxicosis in the field, as well as those experimentally given diclofenac, orally, was severe. Glomeruli, distal convoluted tubules,
and collecting tubules were relatively spared in the vultures that had early lesions. In most vultures, however, lesions became extensive with large urate aggregates obscuring renal architecture. Inflammation was minimal. Extensive urate precipitation on the surface and within organ parenchyma (visceral gout) was consistently found in vultures with renal failure. In addition to the kidney, uric acid crystals were seen most commonly in the liver, spleen, lung, and heart, but were also noted in skin, the adrenal gland, and the parathyroid gland (Meteyer et al., 2005). Swan et al. (2006a) found significant lesions in the kidney, liver, and spleen with extensive uric acid crystal deposition in the vultures treated with diclofenac at 0.8 mg/kg by oral gavage.

Tang (2003) reported that therapeutic use of diclofenac in humans is associated with metabolic idiosyncrasy, which is rare, but may be associated with fatal hepatotoxicity. The onset of clinical signs of this reaction is delayed and there is no clear dose-response relationship.

In dogs receiving methoxyflurane and flunixin meglumine, renal lesions included necrosis of collecting ducts and loops of Henle (Mathews et al., 1990). A somewhat different distribution of lesions was documented by Yasmeen et al. (2007). They found that diclofenac sodium given to adult albino rats in a single daily dose of 2 mg/kg for a period of two weeks caused damage to proximal and distal convoluted tubules with dilation, flattening of tubular epithelium, disruption of brush border in proximal tubule, and thickening of basement membrane around proximal and distal tubular epithelium. Aydin et al. (2003) investigated the histopathologic changes in liver and kidney tissues of albino male Wistar rats treated with diclofenac sodium at the dosage rate of 100 or 150 mg/kg body weight. The liver sections showed cloudy swelling and hydropic degeneration of hepatocytes focal sinusoidal and vena
centralis dilatation, proliferation of bile ducts in portal areas, enlargement of the periportal area with mononuclear cell infiltration, hyperemia and dose-dependent fibrous tissues proliferation and focal necrosis. Kidney tissues exhibited cloudy swelling and hydropic degeneration in the tubular epithelial cells. At either dosage level, necrosis, peritubular lymphocyte infiltration, stromal fibrous tissue proliferation and hyperemia were observed in the kidneys. Rats treated with higher doses of diclofenac (150 mg/kg) developed widespread and intensive necrosis, cloudy swelling, hydropic degeneration, inflammation and increased fibrous tissue in the kidney and liver.

Izumi et al., (1991) conducted experiments for assessment of the carcinogenic potential and the mutagenicity of dipyrone, an antipyretic anodyne in rats pretreated with 0.01% diethylnitrosamine (DEN) in drinking water for two weeks and, after one week of resting, administered 0.4% dipyrone in drinking water, five days a week, for 72 weeks. Hepatocellular carcinomas developed at a higher incidence in the DEN + dipyrone group than in the DEN alone group. Kari et al., (1995) evaluated long-term (2-year studies) toxicity of phenylbutazone, a non-steroidal anti-inflammatory drug in rats and mice. In kidneys, inflammation, papillary necrosis, and mineralization were observed in both sexes of rats, and in females additional lesions included hyperplasia and dilatation of the pelvis epithelium, as well as cysts in kidenys. In male mice exposed to phenylbutazone, males more than females developed hepatic lesions including hemorrhage, centrilobular cytomegaly and karyomegaly, fatty metamorphosis, cellular degeneration, coagulative necrosis, and clear cell foci.

Carrick et al., (1989) reported that foals receiving 6.6 mg/kg of flunixin meglumine, intravenously, for five days, had significantly more gastrointestinal ulceration and cecal petechiation scores than those foals treated with saline. MacAllister et al (1993) examined
the adverse effects of ketoprofen (2.2 mg/kg of body weight), flunixin meglumine (1.1 mg/kg), and phenylbutazone (4.4 mg/kg) in horses dosed i.v. every 8 hours, for 12 days. The glandular portion of the stomach was severely affected by all three NSAIDs. In the phenylbutazone treated group, oedema of the small intestine and erosions and ulcers of the large colon were observed. Renal crest necrosis developed in horses treated with flunixin and phenylbutazone but no renal lesions were observed in ketoprofen-treated group. The toxic potential was greatest for phenylbutazone, less for flunixin meglumine and least for ketoprofen.

Triebskorn et al. (2004) investigated cytopathology in the liver, kidney, gills and gut of rainbow trout (Oncorhynchus mykiss) exposed to five different concentrations (1, 5, 20, 100 and 500 micro-g/L) of diclofenac under laboratory conditions. The most prominent lesions induced by diclofenac were in the kidney, and included, a severe accumulation of protein in the tubular cells (so called hyaline droplet degeneration), macrophage infiltration and structural alterations (dilation, vesiculation) of the endoplasmic reticulum (ER) in the proximal and distal renal tubules. In the liver, the most striking reactions were the collapse of the cellular compartmentation as well as the glycogen depletion of hepatocytes. In the gills, changes included pillar cell necrosis, hypertrophy of chloride cells, and epithelium lifting on the surface of secondary lamellae.

**Blood Chemistry Changes**

It is reported that flunixin meglumine is a very good analgesic for acute and surgical pain in dogs, however, the drug has been associated with increase in alanine amino transferase (ALT) production (Mathews et al., 1996). In a group of dogs undergoing an orthopedic
procedure, the effects on renal function of methoxyflurane anesthesia plus oxymorphone, or of methoxyflurane or halothane anesthesia in combination were assessed. Significant elevations in serum urea and creatinine values, and necrosis of collecting ducts and loops of Henle, were noted in the dogs receiving methoxyflurane and flunixin meglumine (single IM 1.0 mg/kg dose). Hence, the use of combination of methoxyflurane and flunixin meglumine is contraindicated in dogs (Mathews et al. 1990). Cats given flunixin meglumine orally at 1.0 mg/kg for 7 days exhibited an increase in ALT from 11.4 to 21.3 I.U (Taylor et al., 1994). Swinkels et al., (1994) studied the adverse effects of the non-steroidal anti-inflammatory drugs (NSAIDs) ketoprofen (3 mg/kg) and flunixin (2 mg/kg) in pigs after endobronchial challenge with *A. pleuropneumoniae*. Blood parameters were not significantly affected by either NSAID.

Foals treated intravenously with 6.6 mg/kg of flunixin meglumine for five days had no statistically significant blood cellular or biochemical alterations associated with the administration of flunixin meglumine (Carrick et al., 1989). MacAllister et al (1993) reported that horses given phenylbutazone (4.4 mg/kg) i.v. every 8 hours, for 12 days showed a significant decrease in serum total protein and albumin concentration. In ponies, toxic effects of phenylbutazone given at 10 to 12 mg/kg of body weight/day for 8 to 10 days frequently included hypoproteinemia. Plasma loss was usually associated with gastrointestinal ulceration, but sometimes occurred without obvious lesions in mildly affected animals (Snow et al, 1981).

An increase in blood uric acid concentration is often indicative of renal disease and, if moderate to high increases are seen, there may be significant tubule injury (Fudge, 1997). However, normal levels of uric acid may occur in unhealthy kidneys (Hochleithner, 1994).
As a consequence of the large volume of uric acid that is excreted from the tubules independently of the glomerular filtration rate, the concentration of uric acid in the blood is not easily changed (Styles and Phalen 1998). In cases where renal urate deposition occurs prior to visceral gout, anorexia and lethargy may be noted for hours or days. Hyperkalaemia can develop, and this, rather than the uric acid, might lead to cardiac arrest and the sudden death seen with visceral gout (Lierz, 2003).

Pereira and Werther (2007) reported that, in budgerigars (*Melopsittacus undulatus*), the plasma uric acid and protein levels did not change when flunixin meglumine (5·5 mg/kg), ketoprofen (2·5 mg/kg) and meloxicam (0·1 mg/kg) were administered for seven days. Also, no changes in blood uric acid levels were reported in northern bobwhite quails even though they had severe renal lesions resulting from flunixin meglumine treatment (Klein et al, 1994). Significantly elevated creatinine, serum alkaline phosphatase (ALP) and aspartate transaminase (AST) were observed in chickens treated with diclofenac sodium (Reddy et al., 2006). Naidoo et al (2007) observed an increase in serum uric acid concentrations in fowls treated with diclofenac that eventually died. The blood pH gradually decreased to 6.7 at the time of death and this drop in pH corresponded to increases in plasma potassium concentrations.

Swan et al. (2006a) reported increase in plasma concentrations of uric acid and ALT in vultures *Gyps africanus* and *Gyps fulvus* treated with diclofenac 0.8 mg/kg, orally. These birds showed elevated plasma uric acid concentrations, but there was no clear pattern of response for ALT. Plasma uric acid levels at 24 h after diclofenac treatment correlated with the dose administered for *Gyps bengalensis* observed in a previous study (Oaks et al., 2004).
In oriental white-backed vultures (*Gyps bengalensis*), hyperuricaemia was observed 24 hours after treatment with both low and high dosages of diclofenac (Oaks *et al.*, 2004).

**Role of NSAIDs in endotoxemia**

Schwartz *et al.*, (1999) reported that rofecoxib and diclofenac rapidly reversed the elevated temperature in monkeys made febrile by injecting LPS. Specific inhibition of COX-2 by rofecoxib resulted in antipyretic activity in monkeys and humans that was comparable to that of dual COX-1/COX-2 inhibitors such as diclofenac or ibuprofen. The data supported the hypothesis that it is the COX-2 isoform that is primarily involved in the genesis of fever in humans.

Comparative analysis of the impacts of tNSAIDs and selective COX-2 inhibitors suggests that COX-2 is the dominant source of prostaglandins that mediate the rise in temperature evoked by bacterial LPS administration (Mc Adam *et al.*, 1999). Use of NSAIDs in natural as well as induced infections like pneumonia and mastitis is effective in reducing the severity of disease in domestic animals including cattle and horses.

Ewert *et al.*, (1985) studied the effect of dexamethasone, prednisolone and flunixin meglumine on hematologic changes, blood chemical values, and survival in shockin anesthetized ponies given endotoxin (*Escherichia coli* O55:B5) IV. Observed effects of endotoxin included lactic acidosis, prolonged coagulation times, leukopenia, hemoconcentration (A decrease in plasma volume resulting in an increase in the concentration of red blood cells in blood), and elevated blood chemical values. The changes were less severe and survival times were longer in ponies treated with flunixin meglumine.

Olson *et al.*, (1985) studied the effects of endotoxemia on cardiopulmonary parameters in anesthetized ponies. The early increases in mean pulmonary arterial pressure (Ppa),
pulmonary vascular resistance (PVR), and alveolar dead space ventilation (VDA/VT) were blocked by flunixin meglumine (FM), a cyclooxygenase inhibitor. Endotoxin decreased central plasma volume by one hour and cardiac index by three hours; hematocrit and plasma protein concentration were increased by 0.5 and 1.5 hour, respectively, indicating a loss of plasma volume. These changes were also blocked or attenuated by flunixin meglumine. The effects of ketoprofen (2.2 mg/kg) and flunixin meglumine (1.1 mg/kg) on the *in vitro* inflammatory responses of equine peripheral blood monocytes to bacterial endotoxin were compared. There were no significant differences between the effects of these drugs (Jackman *et al.*, 1994). Valk *et al.*, (1998) determined the effect of phenylbutazone on gastric emptying in horses. Phenylbutazone did not alter gastric emptying in normal horses. Nevertheless, while endotoxin caused a profound delay in gastric emptying, pretreatment with phenylbutazone abolished that effect.

Semrad (1993) observed the efficacy of three NSAIDs, flunixin meglumine, ketoprofen and ketorolac tromethamine, for the treatment of endotoxemia in neonatal calves. Although the three NSAIDs prevented eicosanoid production, they provided only partial protection against LPS-induced hypotension. Each NSAID modified responses of the calves to LPS, but none was clearly superior to the others in modulating the clinical signs or physiological alterations induced by infusion of LPS.

Semrad and Dubielzig (1993) studied the effects of phenylbutazone in endoxemia in newborn Holstein calves. Phenylbutazone suppressed the clinical response to endotoxin challenge until large doses (7.5 to 15 micrograms/kg) were administered. Phenylbutazone administration did not increase or ameliorate endotoxin-induced hemostatic alterations or lesions.
Wagner and Apley (2003) determined the effects of two anti-inflammatory drugs (flunixin meglumine and isoflupredone acetate) in lactating Holstein cows with endotoxin-induced mastitis. Neither drug ameliorated loss of milk production or swelling of the affected mammary gland. However, both drugs reduced mean heart rate during the 14 hours following endotoxin administration, compared with untreated control cows. Cows treated with flunixin meglumine had increased rumen motility and decreased rectal temperature during the same period, compared with all other cows. It was previously established that flunixin meglumine provides relief of clinical signs such as fever, local swelling, decreased rumen motility, and lethargy in cattle with mastitis induced by use of *E. coli* or bacterial endotoxin (Anderson *et al.*, 1986a).

Morkoc *et al.* (1993) evaluated effects of the non-steroidal anti-inflammatory agent sodium salicylate on endotoxin-induced mastitis in lactating cows. Although sodium salicylate did not substantially reduce mammary inflammation, it had an antipyretic effect and reduced PGF2α metabolite in mammary blood. Eades (1992) studied administration of flunixin meglumine before infusion of endotoxin at 100 ng/kg in dairy cattle. The drug reduced the effects of endotoxin on both respiratory rate and body temperature.

Andres *et al.* (2000) noticed a significant reduction in the endotoxin-induced clinical signs of inflammation and protein concentration in the aqueous humor in albino rabbits when treated with topical pranoprofen and ophthalmological NSAIDs, i.e. diclofenac and flurbiprofen.

**Efficacy of NSAIDs:**

Toussirot and Wendling (1998) reported that phenylbutazone was considered the NSAID of choice for human patients with ankylosing spondylitis. Muriel-Villoria *et al.*, (1995) compared in a double-blind, double-dummy randomized controlled clinical trial, the onset
and duration of the analgesic effect of dipyrone, 1 or 2 g, and diclofenac sodium, 75 mg, by either the i.m. or the i.v. route in 293 human patients suffering from acute renal colic. The analgesic response seemed more marked and prolonged among patients receiving dipyrone 2 g either i.m. or i.v. However, there were no significant differences between dipyrone (1 g) and diclofenac sodium (75 mg), by either the i.m. or the i.v. route. After dental implant surgery in people, meloxicam and tenoxicam exhibited similar analgesic and anti-inflammatory efficacy (Karabuda et al., 2007). A meta-analysis of comparative randomized clinical trials was carried out to study the efficacy and safety profile of piroxicam, a widely used NSAID by Richy et al. (2009). Regarding global efficacy, piroxicam was more effective than naproxen and nabumetone, while equivalent to other NSAIDs. For pain and articular swelling, piroxicam was statistically equivalent to all other NSAIDs. For mobility, piroxicam appeared to be more effective than indomethacin, while equivalent to all other NSAIDs.

Doig et al. (2000) reported that meloxicam appears to be efficacious in the management of chronic pain associated with osteoarthritis in dogs. They found that meloxicam significantly reduced the clinical signs of chronic locomotive disorders in the dog. Lameness, stiffness, pain on rising, and exercise intolerance all improved in dogs treated with meloxicam. Meloxicam is also efficacious in the management of postoperative pain in dogs (Mathews et al., 2001). Carprofen is a propionic acid-derived NSAID that has anti-inflammatory, analgesic, and antipyretic activity. In animals, carprofen is as potent as indomethacin and more potent than aspirin or phenylbutazone, but carprofen appears to be safer than most other NSAIDs.

Swinkels et al., (1994) studied the antipyretic effect of the non-steroidal anti-inflammatory drugs (NSAIDs), ketoprofen (3 mg/kg) and flunixin (2 mg/kg) in pigs after endobronchial
challenge with *A. pleuropneumoniae*. Ketoprofen showed a highly significant antipyretic effect but flunixin did not. The decrease in food consumption of ketoprofen-treated pigs was significantly less than that of the infected (non-medicated) controls. Hirsch *et al.* (2003) compared the efficacy of meloxicam and flunixin in the treatment of sows with mastitis–metritis–agalactia syndrome. The primary parameter was a clinical index score consisting of rectal temperature, feed intake, general demeanour, respiratory rate, vaginal discharge, degree of inflammation of mammary glands, milk flow and nursing behavior. There was no significant difference between meloxicam and flunixin implying equal efficacy of both drugs. In piglets of diseased litters, however, the mortality rate was 50% lower in the meloxicam group than in the flunixin group in the piglets of sows suffering from mastitis.

The efficacy of ketoprofen in the treatment of acute clinical mastitis in cows in concert with sulphadiazine and trimethoprim was evaluated in a clinical trial (Shpigel *et al.*, 1994). It was concluded that ketoprofen significantly improved recovery in clinical mastitis in dairy cows. A mastitis model was developed by inducing endotoxin in lactating cows which then treated with carprofen. Carprofen reduced heart rate, rectal temperature, and quarter swelling when compared with control cows (Lohuis *et al.*, 1991). The clinical effect of flunixin meglumine administration was determined in cows with acute mastitis induced by intramammary administration of endotoxin. Flunixin meglumine therapy significantly reduced rectal temperatures and signs of inflammation, and improved depression when compared with these signs in saline solution-treated controls (Anderson *et al.*, 1986a). Anderson *et al.*, (1986b) observed the effect of flunixin meglumine in blocking the expected increase in thromboxane concentration in cows with experimentally induced mastitis by intra-mammary infusion of *E.*
coli endotoxin. Flunixin meglumine significantly reduced the production of thromboxane and thus was also able to ameliorate the local inflammation and pain in the mammary gland.

Flunixin meglumine was shown to be effective in the supportive therapy of bovine metritis. In a study involving 259 dairy cows that had postpartum metritis from 21 different dairy farms in Greece (Amiridis et al., 2001), cows receiving flunixin meglumine had lower rectal temperatures, faster uterine involution, and a significantly shorter calving-to-first estrus interval than those that did not receive flunixin. In a model of naturally occurring bovine mastitis (Dascanio et al., 1995), phenylbutazone was not as effective as flunixin meglumine in mediating the signs of endotoxemia. Banting et al. (2008) studied the efficacy of oral and parenteral ketoprofen in lactating cows with LPS-induced acute mastitis. Ketoprofen administered either orally or parenterally significantly reduced the effects of the LPS on rectal temperature, ruminal contractions, and respiratory rate. The udder swelling and the signs of pain were also reduced.

In calves with bronchopneumonia induced by LPS, a significant reduction in the main inflammatory mediators PGE2, PGF2α, thromboxane (TXB2), and malonyldialdehyde (MDA) was observed when treated with meloxicam compared to the positive controls. Contrary to effects observed by flumethasone, meloxicam induced an increase of leukotriene B4 (LTB4) and interferon-alpha (INFα) indicating that it is not immunosuppressive (Bednarek et al., 2005).

The treatment of black-and-white lowland breed calves with clinical signs of enzootic bronchopneumonia with oxytetracycline in combination either with meloxicam or flunixin, meglumine produced a significantly faster, in comparison to the control group, normalization of body temperature (Bednarek et al., 2003a). The combinations of meloxicam or flunixin...
meglumine with an antibiotic were equally effective in the treatment of calves and superior to the antibiotic alone. Similarly, the clinical efficacy of meloxicam and flunixin meglumine as adjuncts to oxytetracycline 20 mg/kg in the treatment of acute febrile respiratory disease (bronchopneumonia) in cattle were compared (Friton et al., 2004). A significantly lower mean rectal temperature was measured in the meloxicam group and a single subcutaneous dose of meloxicam was as clinically effective as up to 3 consecutive daily intravenous doses of flunixin meglumine. Van de Weerdt et al., (1999) compared the effect of two NSAIDs, phenylbutazone and ketoprofen in a model of PAF-induced reversible lung inflammation in calves. ketoprofen (3 mg/kg, IM) was more effective than phenylbutazone (10 mg/kg, IM) in preventing respiratory dysfunctions induced by the platelet-activating factor (PAF)-challenge at 30 min after drug administration.

In Holstein calves that had an experimentally-induced pneumonia by Mannheimia hemolytica, NSAIDs were more beneficial than corticosteroids as supportive treatment in the associated bovine respiratory disease (Bureau et al., 1998). In naturally affected calves with acute respiratory disease, a single dose of carprofen was as effective in alleviating clinical signs as three daily doses of flunixin meglumine. There were no significant differences between the NSAID groups in reduction of clinical signs (Balmer et al., 1997). The long-term effects of a single dose of meloxicam in conjunction with antibiotic therapy in cattle with clinical signs of bovine respiratory disease (BRD) were evaluated (Friton et al., 2005). The mean bodyweight, the mean average daily weight gain and the mean carcass weight of the animals treated with meloxicam were significantly higher and there were fewer and less severe lung lesions. Lockwood et al. (2003) compared the relative efficacy of flunixin, ketoprofen and carprofen, in conjunction with ceftiofur, in the treatment of naturally
occurring bovine respiratory disease. There was no significant difference was found among
the treatment groups with respect to depression, illness scores, dyspnoea or coughing. There
was less lung consolidation in the three groups treated with a NSAID than in the animals
treated with ceftiofur alone, but the difference was significant only in the group treated with
flunixin. Deleforge et al., (1994) compared the treatment of respiratory disease in cattle with
oxytetracycline alone to a combination of oxytetracycline and tolfenamic acid. The animals
given the combination therapy had improved clinical signs, weight gain, and fewer relapses.
Also, a trial was conducted in 4- to 5-week old
Raza et al., (2000) compared different protocols (combinations of antimicrobials and
NSAIDs) for the treatment of haemorrhagic septicaemia in buffaloes and cattle. Treatment
with norfloxacin and diclofenac sodium was more effective than the treatment with
gentamicin and dipyrone.
Constable (2009) recommended ancillary treatment with NSAIDs such as meloxicam and
flunixin meglumine, and continued feeding of cow's milk as an adjunct treatment of diarrhea
in calves with systemic signs of illness, manifested as fever, inappetance and lethargy.
Barnett et al. (2003) assessed the use of flunixin meglumine at the dosage rate of 2.2 mg/kg
body weight, as an adjunct treatment for diarrhea in calves. Calves with diarrhea containing
fecal blood that were treated with a single dose of flunixin meglumine had fewer morbid
days and antimicrobial treatments, compared with controls. The effects of ketoprofen on
Escherichia coli heat-stable enterotoxin-induced diarrhea in calves were studied by Roussel
et al.(1993). It was reported that fecal output was less at 8 hours and 24 hours for calves
given ketoprofen (6 mg/kg) compared with the control day.
Dehorning and disbudding are routine painful procedures carried out on cattle to facilitate management. Stafford and Mellor (2005b) reported that use of ketoprofen along with cornual nerve blockade with lignocaine and use of xylazine before cautery disbudding given before and after disbudding optimally relieves the pain. The behaviour of calves treated with combination therapy suggested that pain was alleviated for 24 h. Milligan et al. (2004) investigated the value of ketoprofen in reducing the pain following dehorning. They found that ketoprofen, in addition to local anesthesia, may alleviate short-term pain following dehorning with a butane dehorning device in dairy calves less than two weeks of age.

Faulkner & Weary (2000) found that ketoprofen mitigates pain after hot-iron dehorning in young Holstein dairy calves when used in addition to sedative (xylazine) and local anaesthetic (lidocaine) before dehorning and also tended to gain more weight than did control calves. The results of a study by McMeekan et al. (1998), suggest that use of ketoprofen with lignocaine (regional analgesia) reduce plasma cortisol concentration indicating reduction in distress associated with dehorning in calves. Stewart et al. (2009) studied the effects of meloxicam on physiological responses of calves immediately after hot-iron dehorning and during the time that local anesthetic (LA) wears off (2 to 3 h) after this procedure. This study demonstrated that the combination of LA and NSAID mitigated the onset of pain responses when the LA wanes.

The impact of meloxicam on post-surgical stress associated with cautery dehorning in Holstein heifer calves was studied by Heinrich et al. (2009). The changes in heart rates and respiratory rates were greater in the control group compared with the meloxicam group. The results indicated that meloxicam reduced the physiological stress response to dehorning.
Castration is an ancient husbandry procedure used to produce docile cattle for draught work, to reduce unwanted breeding, and to modify carcass quality, but all the physical methods used to castrate cattle have side-effects and cause pain. When used alone, ketoprofen sometimes reduces the cortisol response to Burdizzo or surgical castration, but may need to be accompanied by local anaesthesia to eliminate the pain-induced behaviour seen during the castration process itself (Stafford and Mellor, 2005a). The effects of burdizzo castration alone or in combination with ketoprofen (K), local anesthesia (LA), or caudal epidural anesthesia (EPI) in Holstein x Friesian bulls were evaluated by Ting et al. (2003). They noticed that the use of K or EPI was more effective than LA in decreasing pain-associated behavioral responses observed during the first 6 h after treatment.

Ting et al. (2003) studied the effects of repeated ketoprofen administration during surgical castration of bulls on cortisol, immunological function, feed intake, growth, and behavior. It was reported that systemic analgesia with ketoprofen is an effective method for alleviating acute inflammatory stress associated with castration. The cortisol responses of calves to different methods of castration (ring, band, surgical, clamp) were studied with or without local anaesthetic, or local anaesthetic plus a NSAID (Stafford et al., 2002). This response to surgical castration, by traction on the spermatic cords or by cutting across them with an emasculator, was eliminated when ketoprofen was given with the local anaesthetic.

In Friesian calves surgical castration induced a significant elevation in cortisol secretion; the rise in cortisol was reduced to control levels by the administration of ketoprofen but not local anaesthetic (Earley & Crowe, 2002).
The effect of ketoprofen in the impaired gait associated with pain in lactating Holstein cows was evaluated by Flower et al. (2008). The numerical rating system (NRS) improved by 0.25 +/- 0.05 with the highest dose of ketoprofen i.e. 3 mg/kg body weight. However, ketoprofen had only a modest effect on gait, indicating either that this drug has little effect on pain due to lameness or that much variation in NRS was due to factors other than pain. No significant effect of ketoprofen was observed in reducing the locomotion score in lame dairy cows (Whay et al., 2005). Instead, hyperalgesia associated with lameness was recorded using a nociceptive threshold test in the cows that received ketoprofen.

In a study with horses designed to find the median effective dose (ED50) of meloxicam to reduce lameness score, it was suggested that meloxicam acted as a potent anti-inflammatory drug, and a dosage of 0.6 mg/kg/d would be appropriate for use in a clinical study (Toutain and Cester, 2004). The analgesic and anti-inflammatory effects of ketoprofen and phenylbutazone were compared in an acute equine synovitis model (Owens et al., 1996). Phenylbutazone was more effective than ketoprofen in reducing lameness, joint temperature, synovial fluid volume, and synovial fluid PGE2. The anti-inflammatory properties of flunixin (2.2 mg/kg) and ketoprofen (2.2 mg/kg) were studied in a model of acute inflammation, comprising surgically implanted subcutaneous tissue cages stimulated by intracaveal injection of carrageenan in horses (Landoni and Lees, 1995). Both the drugs inhibited bradykinin-induced swelling. Roelvink et al., (1991) evaluated the analgesic and spasmolytic effects of dipyrone (Novalgin) (2500 mg/100 kg bodyweight), hyoscine-N-butylbromide (Buscopan) (20 mg/100 kg bodyweight) and a combination of both drugs in a balloon-induced model of colic, using five ponies with caecal fistulae. The results on pain relief were not statistically significant for any of the drugs.
The effects of etoricoxib, piroxicam, indomethacin, as well the combination of etoricoxib either with piroxicam or indomethacin, were evaluated on articular incapacitation and edema in rats. Etoricoxib, piroxicam, and indomethacin inhibited incapacitation and edema (Bressan and Tonussi, 2008).

Summary:

Non-steroidal anti-inflammatory drugs are the group of compounds used to alleviate pain, fever and inflammation. These drugs act by inhibiting the cyclooxygenase enzymes and thus inhibit the synthesis of prostaglandins. By the same mechanism, the synthesis of some physiologically important prostaglandins is also inhibited, and this can result in adverse effects associated with these drugs. Many adverse effects are attributed to these drugs in humans and animals, including gastrointestinal ulceration, renal damage and allergic reactions. Diclofenac, a member of this class of drugs is being used in humans for the treatment of a variety of pathological conditions including osteoarthritis, dental pain and various types of colic. This drug uniquely harmed South Asian vultures and caused heavy causalities in this species. It was observed that the vultures developed visceral gout after ingestion of the meat of dead animals that had been treated with this drug shortly before their death. After several investigations it was recommended that the use of diclofenac in domestic animals should be banned. It was essential to search for an alternative drug that would be safe for the scavenging birds and as efficacious in treating domestic animals as diclofenac. Keeping in view this dire need the present project was undertaken.
Chapter 3
MATERIALS AND METHODS

Experiment No. 1:

DEVELOPMENT OF CHICKEN EXPERIMENTAL MODEL TO STUDY THE TOXICITY OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

Experimental Birds

One hundred, one-day old broiler chicks (Gallus gallus domesticus) were purchased from the “Big Bird” Hatchery and reared on a floor covered with litter (wood shavings) in the Experimental Sheds of the Department of Pharmacology and Toxicology, University of Veterinary and Animal Sciences, Lahore, Pakistan. They were provided with commercially available broiler chick starter ration No. 4 and fresh clean water *ad libitum* until the end of experimental period. The house temperature was maintained at 35°C during first week of their life. The temperature was then reduced 2°C each week until it reached to 25°C, which was maintained for the rest of the study period. Birds were vaccinated against important infectious diseases according to the vaccination schedule shown in Table 3.1 (Haq, 2004).

Table 3.1: Vaccination schedule for the experimental broiler chickens.

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>Vaccine</th>
<th>Dosage</th>
<th>Route</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>New Castle Disease Vaccine (NDV)</td>
<td>0.025ml</td>
<td>Eye Drops</td>
</tr>
<tr>
<td>10</td>
<td>Infectious Bursal Disease Vaccine (IDBV)</td>
<td>0.025ml</td>
<td>Eye Drops</td>
</tr>
<tr>
<td>16</td>
<td>Hydropericardium Syndrome Vaccine (HPSV)</td>
<td>0.5ml</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>20</td>
<td>IBDV</td>
<td>0.5ml</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>22</td>
<td>NDV</td>
<td>0.5ml</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>28</td>
<td>IBDV</td>
<td>0.5ml</td>
<td>Intramuscular</td>
</tr>
</tbody>
</table>
No medication was given to the experimental birds except the test drug i.e. diclofenac. Birds were kept and handled according to the guidelines framed by the Animal Usage and Care Committee of the University.

**Experimental Design**

On day thirty-five, these birds were randomly divided into two main groups, A and B and then each group was further divided into five subgroups. The birds in subgroups A1, A2, A3 and A4 were treated with diclofenac sodium at the dose of 1.25 mg/kg, 2.5 mg/kg, 5 mg/kg and 10 mg/kg body weight respectively, intramuscularly (pectoral muscles), once daily for four consecutive days. The volume of the injection was adjusted to 0.2 ml/kg body weight in order to deliver the same dose volume for each bird. The birds in subgroup A5 were injected with 0.2 ml/kg of physiological normal saline (PNS) solution intramuscularly and served as controls. The birds in subgroups B1, B2, B3 and B4 were treated with diclofenac sodium in the similar manner as mentioned above but via the oral route. The birds in subgroup B5 (Control) were given PNS solution at the rate of 0.2 ml/kg, orally.

**Drugs and Chemicals**

Diclofenac sodium injection, 50mg/ml. (Diclostar, manufactured by Star Laboratories, Pakistan).

Physiological normal saline (PNS) solution

Serum uric acid, creatinine, AST, ALT and ALP measuring/diagnostic kits (Randox, UK)

Sodium phosphate monobasic

Sodium phosphate dibasic, anhydrous

Formaldehyde 10% natural buffered

Paraffin

Hematoxylin and eosin stains

**Equipment**

Disposable syringes (1 ml, insulin) were used for injection of drugs.
Vacutainer tubes (2 ml) were used for the collection of blood samples.
Centrifuge was used for separation of serum from blood samples.
Eppendorff tubes (serum cups) were used for storage of serum samples.
Spectrophotometer (UV-1650PC, Shimadzu Corporation, Japan) was used for serum biochemical analysis.
Plastic jars were used for collection of tissue samples for histopathology.
Paraffin blocks were used for embedding for sectioning for histopathology

Experimental Protocol

Drug Treatment
The birds in group A were injected with PNS solution at the rate of 0.2 ml/kg body weight. The birds in groups B, C, D and E were treated with diclofenac sodium at the dose of 1.25 mg/kg, 2.5 mg/kg, 5 mg/kg and 10 mg/kg body weight, respectively. The volume of injection was adjusted to 0.2 ml/kg b.w. by adding PNS in the drug solution. The injection was given daily, intramuscularly in the pectoral muscles using each side on alternate days, for four days.

Clinical Parameters
The birds in all groups were examined twice daily for their feeding, drinking and general appearance. Abnormalities in behavior, such as evidence of depression, change in body movement, posture, or feed and water intake, were recorded.

Mortality
Mortality rates in different dosage groups were observed and recorded throughout the experimental period and compared to evaluate the toxicity of the test drug.
**Blood Sampling**

Two ml blood was collected from each bird using vacutainer tubes from the right jugular vein. Blood was collected before the start of experiment and then after every 24 hours up to 96 hours. The blood samples were centrifuged for 10 minutes at 1500 rpm, serum was separated in serum cups and stored at -20°C for further analysis.

**Postmortem Examination**

Postmortem examination was performed on birds that died during the experimental period as soon as possible after their death as well as upon euthanasia of all birds that survived to the end of observation period. Gross lesions in the external and internal organs were observed and recorded.

**Biochemical Analysis**

**Analysis of Urates**

The white substance accumulated in the pericardium was collected and analyzed via the Murexide Test. Urate deposits were mixed with nitric acid and dried over a flame. A drop of concentrated ammonia was added, and development of a mauve color confirmed the presence of uric acid (Lumeij, 1994).

**Biochemical Analysis**

Serum samples separated from the blood samples collected at different time intervals were analyzed for the levels of uric acid and creatinine, and the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). The following procedures were used for the estimation of afore-mentioned assays.

**Uric Acid**

Serum uric acid was measured by a colorimetric method (Fossati et al, 1980).

**Principle**

Uric acid was measured after conversion by uricase to allantoin and hydrogen peroxide, which under the catalytic influence of peroxidase, oxidizes 3,5-dichloro-2-
hydroxybenzenesulphonic acid and 4-aminophenazone to form a red-violet quinoneimine compound.

Uric Acid$+\text{O}_2+2\text{H}_2\text{O}\rightarrow \rightarrow \rightarrow \text{Allantoin}+\text{CO}_2+\text{H}_2\text{O}_2$

$2\text{H}_2\text{O}_2+3,5\text{-dichloro-2-hydroxybenzenesulphonic acid}+4\text{-aminophenazone}\rightarrow \rightarrow \rightarrow N-(4\text{-antipyryl}-3\text{-chboro-5-sulfonate-p-benzo-quinoneimine}).$

**Sample:** Serum free of hemolysis.

**Standard:** Standard solution containing uric acid 10 mg/ml. different dilutions were made as 0.1, 1 and 10 mg/ml to establish calibration curve.

**Reaction Requirements**

- **Wavelength** 520 nm
- **Cuvette** 1 cm light path
- **Reaction temperature** 25°C

**Measurement against Reagent Blank**

The tubes were labeled as Sample, Standard and Reagent Blank. A volume of 20 µl each of serum sample and the uric acid standard was pipetted into the respective test tubes and 1 ml reagent was added to all three tubes. The solutions were mixed and incubated for 15 min. at 25°C. The absorbance of sample ($A_{\text{sample}}$) and the standard ($A_{\text{standard}}$) were measured against the reagent blank. Serum uric acid concentration was calculated as:

$$\text{Uric acid (mg/dl)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times 10.$$  

Accuracy and reproducibility were monitored by using assayed Multi-Sera for low, normal and elevated uric acid values.

**Creatinine**

Serum creatinine was measured using a colorimetric method described by Bartels and Bohmer, 1972.
MATERIALS AND METHODS

**Principle**

Creatinine in alkaline solution reacts with picric acid to form a colored complex. The amount of the complex formed is directly proportional to the creatinine concentration.

**Reaction Requirements**

Wavelength 492 nm  
Cuvette 1 cm light path  
Reaction temperature 25°C  

**Measurement against Air**

Solutions 2 and 3 contained picric acid and sodium hydroxide, respectively. Equal volumes of these solutions were mixed to prepare the working reagent. One ml of the working reagent was placed in Standard and Sample tubes, and 0.1 ml of standard and samples were then added into the respective test tubes. The solutions were mixed and absorbance (A1) was taken for standard and sample after 30 seconds. The second absorbance (A2) was read after 2 minutes for standard and sample against air.

**Calculation of Creatinine in Serum**

\[ A2 - A1 = \Delta A_{\text{sample}} \text{ or } A_{\text{standard}} \]

Serum creatinine (mg/dl) = \( \frac{A_{\text{sample}}}{A_{\text{standard}}} \times 2 \)

**Aspartate aminotransferase (AST)**

The colorimetric method of Reitman and Frankel (1957) was used for the determination of serum aspartate aminotransferase activity.

**Principle**

When α-oxoglutarate combines with L-aspartate in the presence of AST, L-glutamate and oxaloacetate is formed. The oxaloacetate further reacts with 2,4-dinitrophenyl-hydrazine (2,4-DNP) resulting in the formation of oxaloacetate hydrazone. The concentration of the resultant compound is monitored to indirectly measure AST activity in the serum.
Reaction Requirements

Wavelength  546 nm
Cuvette  1 cm light path
Incubation temperature  37°C

Measurement against Reagent Blank

The test tubes were labeled as Reagent Blank and Sample. Volumes of 0.1 ml sample and distilled water were added in each tube respectively. Then 0.5 ml buffer was added in both tubes. The solutions were mixed and incubated for exactly 30 min. at 37°C. A volume of 0.5 ml of 2,4-DNP was added to the tubes and allowed to stand for exactly 20 min. at 20-25°C. Lastly, 5ml of sodium hydroxide solution were added and mixed in each tube, and absorbance of sample (A_{sample}) was read against the reagent blank after 5 minutes.

The enzyme activity (U/L) in serum samples was obtained from the table showing the absorbance vs. activity, provided along with the Reagent Kit. Assayed Multi-Sera for low, normal and elevated were used for quality control.

Alanine Aminotransferase (ALT)

A colorimetric method (Reitman and Frankel, 1957) was used for the determination of serum aspartate aminotransferase activity.

Principle

L-alanine reacts with α-oxoglutarate in the presence of ALT to form L-glutamate and pyruvate. Pyruvate hydrazone is produced when pyruvate reacts with 2,4-DNP. Measurement of serum ALT was done by monitoring the concentration of pyruvate hydrazone.
Reaction Requirements

Wavelength  546 nm
Cuvette  1 cm light path
Incubation temperature  37°C

Measurement against Sample Blank

The test tubes were labeled as Sample and sample Blank. Each serum sample was poured into a Sample tube, buffer solution was added to both the tubes. Each tube was mixed and then incubated at 37°C for exactly 30 min. A volume of 0.5 ml 2,4-DNP was added in both tubes and 0.1 ml serum sample in sample Blank tube. After mixing, the solutions were allowed to stand for exactly 20 min. at 25°C. Then 5 ml of the sodium hydroxide solution were mixed with both tubes, and absorbance of the sample \((A_{\text{sample}})\) against the sample blank was taken after 5 minutes. The enzyme activity \((U/L)\) of serum samples was obtained from the table showing the absorbance vs. activity, supplied along with the Reagent Kit. Assayed Multi-Sera for low, normal and elevated were used for quality control.

Alkaline Phosphatase (ALP)

Serum alkaline phosphatase was measured by an optimized standard colorimetric method (Rec. GSCC, 1972).

Principle

When p-nitrophenylphosphate reacts with water in the presence of ALP it produces phosphate and p-nitrophenol. The amount of the product formed as detected by UV absorbance reflects the activity of ALP in the sample.
Reaction Requirements

Wavelength  405 nm
Cuvette  1 cm light path
Incubation temperature  37°C

Measurement against Air

Each serum sample (0.01 ml) and reagent (0.5 ml) was mixed and poured in the cuvette. The initial absorbance was taken immediately and after 1, 2, and 3 minutes against air.

Calculations

Serum ALP activity (U/L) = 2760XΔA

The quality control was monitored using assayed Multi-Sera.

Histopathology:

Collection and Fixation of Tissues

Tissue samples were collected during postmortem examinations for histopathology from the birds that died and from the birds that were killed at the end of experiment. Specimens of kidneys, liver, heart, spleen and skeletal muscles of the dosed and control birds were obtained for comparative histopathology. The 10% neutral formalin solution was prepared by adding 100 ml formaldehyde, 4 g of sodium phosphate monobasic and 6.5 g sodium phosphate dibasic (anhydrous) in distilled water to make the volume 1000 ml. For this purpose, the phosphate buffers were first dissolved in hot distilled water and then the formaldehyde was added to avoid vaporization. The container was inverted several times to ensure proper mixing. The tissues were sliced at a thickness of 5 mm and placed in at least ten times more volume of 10% neutral buffered formalin solution than that of the tissue in the plastic jars. The containers were stirred to enhance the penetration of fixative in the tissue. The tissues specimens remained in the fixative for 48 hours before further processing to ensure proper fixation.
Tissue Processing

The following steps were performed to prepare the tissue sections for microscopic examination;

Dehydration

The fixed tissues were passed through ascending concentrations of alcohol and finally through absolute alcohol to completely remove water.

Clearing

The dehydrating agent, alcohol, is not miscible with the impregnating medium (paraffin wax). Accordingly, xylene, which is miscible with both alcohol and paraffin, was used to remove the alcohol.

Embedding

The tissues after clearing were embedded in paraffin wax. For this purpose the molten wax was dispensed into the moulds and then the tissues were placed at the bottom.

Tissue Sectioning and Preparation of Slides

After the tissue blocks solidified, they were trimmed and fixed on a microtome for sectioning. Sections of 5 µm thickness were cut to make a ribbon of the tissue sections. The ribbons then placed on the surface of water in a hot water bath adjusted at a temperature near the melting point of paraffin. The tissue sections were lifted on the glass slides already smeared with methyl cellulose, an adhesive material. These slides were dried on the hot plate regulated at the temperature above the melting point of paraffin.

Staining of the Slides

The slides with tissue sections were cleared of paraffin by using xylene. The xylene was removed by using alcohol. Then the slides were passed through descending concentrations of alcohol to rehydrate the tissues. This was necessary because hematoxylin and eosin (H&E) stain works in aqueous medium. The tissue sections were stained with H&E Stain according to the procedure described by Bancroft and Steven (1990).
Microscopic Examination of Slides

The stained tissue slides were examined under a light microscope at different magnification levels to observe the histopathological changes (Bancroft and Steven, 1990). These slides were micrographed for further exploration and as a future record.

Statistical Analysis

Mortality percentages in the different groups were compared. The data regarding serum uric acid, creatinine, AST, ALT and ALP were analyzed by two way analysis of variance. The difference among various treatment groups was calculated using the Least Significant Difference (LSD) test ($p<0.05$) (Steel and Torrie, 1982).
Experiment No. 2:

COMPARATIVE TOXICITY OF DICLOFENAC IN DIFFERENT AVIAN SPECIES

In a previous experiment, it was observed that diclofenac was toxic to broiler chicken, hence two other species (Japanese quail and pigeon) were included in this experiment to assess potential species variation in toxicity of this drug. Quail (*Coturnix coturnix japonica*) and pigeon (*Columbia livia*) were selected due to the similarities of these species to broiler chickens. Twenty chicks of each species, i.e. broiler chicken, Japanese quail and pigeon, were reared for 60 days on the University’s Quail Experimental Farms, Lahore. Feed and water were provided to these birds *ad libitum*. The birds of each species were randomly divided into two groups, i.e. control (C) and treatment (T).

**Drugs and Chemicals**

Diclofenac sodium, physiological normal saline solution and diagnostic kits for the estimation of uric acid, creatinine, AST, ALT and ALP were obtained from the same sources as mentioned in experiment no.1.

**Study Protocol**

The birds in the treatment group of each species were given diclofenac sodium at the dose of 10 mg/kg body weight, whereas the birds in the control groups received an injection of physiological normal saline solution. The dose of diclofenac sodium was selected on the basis of the results of experiment no. 1. The volume of injection was adjusted at 0.2 ml per kg body weight by adding PNS, where required as described in experiment no. 1. The treatment was given intramuscularly, once daily and continued for four consecutive days.

**Equipment**

The equipment used in experiment no. 1 was also utilized in blood and tissue sample collection from the birds in this experiment.
MATERIALS AND METHODS

Blood Sampling

Blood samples were collected from all the birds before the start of experiment. Blood samples from chickens and pigeons were drawn from the right jugular vein and those from quails directly from heart. The affected birds were also sampled before death during the experiment and from the euthanized birds at the end of the observation period. Blood collection and serum separation procedures were the same as described in experiment no.1.

Clinical Observations and Mortality

The birds in all groups were observed for behavioral and physical changes twice a day during the experimental period. The clinical abnormalities and mortality in each group were recorded carefully.

Postmortem Examination

Necropsies were performed on the dead birds soon after their death and on those killed at the end of experiment. Gross lesions found in external and internal organs were recorded.

Histopathology

Samples of kidneys, liver, heart, spleen and skeletal muscles were collected from dead or euthanized birds, including those from the control group for each species. The collection, processing and staining of these tissue samples were conducted according to the standard procedures explained in the previous experiment.

Serum Biochemical Analyses

Serum samples separated from the blood collected from experimental birds were processed for the estimation of serum uric acid, creatinine, AST, ALT and ALP following the prescribed procedures as mentioned in experiment no. 1.
Statistical Analysis

Percent mortality rates in chicken, pigeon and quail after dosing with diclofenac sodium were compared to observe differences in susceptibility among these species. The data obtained from serum biochemical analyses at different time intervals for uric acid, creatinine, AST, ALT and ALP in different groups were analyzed through two way analysis of variance. The difference among various treatment groups was calculated using LSD (p<0.05) (Steel and Torrie, 1982).
Experiment No. 3:

SCREENING OF NONSTEROIDAL ANTIINFLAMMATORY DRUGS REGARDING THEIR TOXICITY IN THE CHICKEN MODEL

In this experiment, various NSAIDs were studied to establish their toxicity profiles in the chicken experimental model.

Experimental Birds

For this purpose eighty, one-day-old broiler chicks were reared in the Experimental Sheds of the Department of Pharmacology and Toxicology, University of Veterinary and Animal Sciences, Lahore, Pakistan. The experimental birds were maintained, vaccinated and fed according to the procedures mentioned in experiment no. 1.

Drugs and Chemicals

The following medicines were used in this experiment:

1) Piroxicam U.S.P (Rumolon; TabrosPharma) injection 20 mg/ml.
2) Ketoprofen (Profenid; Aventis Pharma) injection 50 mg/ml.
3) Phenylbutazone (Butadin; Star Laboratories, Lahore) injection 200 mg/ml.
4) Metamizole (Dipyron; Orient Labs. Pvt. Limited) injection 250 mg/ml.
5) Meloxicam (Melonex; Intas Pharmaceutical, India) injection 5 mg/ml.
6) Flunixinmeglumine (Loxin; Selmore Pharmaceutical, Pakistan) injection 50 mg/ml.
7) Diclofenac sodium (Diclostar; Star Laboratories, Pakistan) injection 50 mg/ml.
8) Physiological Normal Saline injection.
Experimental Protocol

On day 35, the birds were randomly divided into eight groups with ten birds in each group. The birds in groups one to six were treated with phenylbutazone (PBZ), metamizole (MTZ), piroxicam (PX), meloxicam (MX), ketoprofen (KP) and flunixin meglumine (FM). The seventh group was treated with diclofenac (DF) and served as a reference. The birds of group eight were given physiological normal saline (PNS) solution and kept as the control. The treatment was repeated after each 24 hours and continued up to four days. The injection was given in pectoral muscles alternating between sides. The dose of each drug was set at four times the therapeutic dose. The logic behind this is that the signs of toxicosis and mortality were evident at this dose when diclofenac was studied in the previous experiments. The volume of the injection was uniform (0.5 ml/kg b.w.) for all the treatment and control groups. The following doses of these drugs were injected to the experimental birds in the respective groups:

Table 3.2: Various treatment groups of broiler chickens and dosages of NSAIDs for the screening of their toxicity.

<table>
<thead>
<tr>
<th>Group #</th>
<th>Drug Administered</th>
<th>Dosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Phenylbutazone (PBZ)</td>
<td>100 mg/kg body weight</td>
</tr>
<tr>
<td>2.</td>
<td>Metamizole (MTZ)</td>
<td>100 mg/kg “ “</td>
</tr>
<tr>
<td>3.</td>
<td>Piroxicam (PX)</td>
<td>2 mg/kg “ “</td>
</tr>
<tr>
<td>4.</td>
<td>Meloxicam (MX)</td>
<td>2 mg/kg “ “</td>
</tr>
<tr>
<td>5.</td>
<td>Ketoprofen (KP)</td>
<td>10 mg/kg “ “</td>
</tr>
<tr>
<td>6.</td>
<td>Flunixin meglumine (FM)</td>
<td>10 mg/kg “ “</td>
</tr>
<tr>
<td>7.</td>
<td>Diclofenac (DF)</td>
<td>10 mg/kg “ “</td>
</tr>
<tr>
<td>8.</td>
<td>Physiological normal saline (PNS)</td>
<td>0.5 ml/kg “ “</td>
</tr>
</tbody>
</table>
Study Parameters:

Clinical Signs and Mortality

The experimental birds were observed for clinical signs and behavioral changes twice a day at 12 hour intervals throughout the observation period. All such changes were recorded for comparison among the treatment groups.

Postmortem Examination

A complete postmortem examination was conducted on the dead birds in the FM and DF groups immediately after death. Necropsy was also performed immediately after euthanasia of the surviving birds of these groups, birds in other treatment groups, and those in the control group. The external features examined for abnormalities included skin, feathers, muscle mass, eyes, nostrils, ears, wings, feet, cloaca and vent. Gross internal observations were made on pectoral muscles, thoracic and abdominal air sacs, heart and major blood vessels, thyroid and adrenal glands, brain, liver, kidneys, spleen, lungs, trachea, intestinal tract, reproductive organs, and tibiotarsal, metatarsal and digital joints.

Blood Collection

Blood samples were drawn from all experimental birds before commencement of the experiment and then after every 24 hours until the end of the observation period following the procedure described in experiment no.1. Serum separation and storage were done adopting the already explained methods.

Biochemical Analyses

Serum separated was analyzed for the estimation of uric acid, creatinine, AST, ALT and ALP by standard procedures as described above.
Histopathology

Tissue samples from kidney, heart, liver, spleen and skeletal muscles were collected from the birds during postmortem of dead and killed birds, processed, and examined for histopathological changes, using the methods described in experiment no. 1.

Statistical Analysis

The experiment was designed using a completely randomized design with factorial arrangement. The data regarding mortality in different treatment groups were compared after calculating the mortality percentage. The data obtained from serum biochemical analysis at different time intervals were compared by using two way ANOVA and significant differences among the groups at different time points were identified using LSD (p<0.05) (Steel and Torrie, 1982).
EXPERIMENT No. 4

Efficacy Trials of Nonsteroidal Anti-inflammatory Drugs in Experimentally Induced Pyrexia in Buffalo Calves

After screening a number of NSAIDs in broiler chickens for their toxicity profile, meloxicam, piroxicam and ketoprofen were selected for experimental evaluation in buffalo calves, one of the target species. The drugs were evaluated for their efficacy against pyrexia induced by *E. coli* lipopolysaccharide (LPS), and their efficacy was compared with that of diclofenac.

**Experimental Animals**

Thirty Nili-Ravi buffalo calves (*Bubalus bubalis*), 9 to 12 months of age with body weights ranging from 98 to 114 kg, were selected and maintained at the Livestock and Dairy Development Department’s Animal Nutrition Centre, Experimental Research Station, Rakh Dera Chahal in Lahore, Pakistan. These animals were randomly divided into six groups having 5 animals in each group. Each group was kept in separate pens and supplied with the ration formulated by the Centre for routine feeding of these animals. Fresh, clean drinking water was provided to the animals of all the groups, *ad libitum*. These animals were acclimatized for a week before the start of experiment.

**Drugs and chemicals:**

1) LPS (*E. coli* 055:B5, Cat. No. 62326 *BioChemica*, Fluka, USA). LPS was dissolved in sterile PBS solution at a concentration of 2.5 µg/ml and filtered by a syringe filter of 0.2 µm pore size (Sartorius Stedim).

2) Diclofenac sodium, 50 mg/ml (Diclostar, Star Laboratories, Pakistan).

3) Ketoprofen 50 mg/ml (Profenid, Aventis)

4) Piroxicam 20mg/ml (P cam, Merck)
5) Meloxicam 5 mg/ml (Melonex, Intas Pharmaceuticals, India)

**Study Design:**

All the animals were ear tagged and clinically examined for any abnormality before the start of experiment. These calves were randomly divided into six groups, containing five animals in each group. Animals in group 1 remained unexposed to LPS and served as environmental controls. They were administered physiological normal saline (PNS) solution. Group 2 animals were untreated positive (LPS) controls, while the animals in groups 3 to 6 were treated with LPS and different NSAIDs.

**Table 3.3: Treatment regimens administered to various groups of experimental buffalo calves.**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Treatment Regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>Normal Saline (NS)</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>Lipopolysaccharide (LPS) + NS</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>LPS + Diclofenac sodium</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>LPS + Ketoprofen</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>LPS + Meloxicam</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>LPS + Piroxicam</td>
</tr>
</tbody>
</table>

A winged-blood sampling set (MN*SVS 21 BQ 30, Terumo Europe Lab Systems) was inserted into the jugular vein and fixed properly in each animal, 24 hours before the injection of LPS and collection of blood, to allow the animals to become somewhat accustomed to the procedure. Animals in group 1 were given PNS, at 50 ml per animal intravenously, while the animals in groups 2 to 6 were injected with LPS at the dose rate of 1 µg/kg body weight i.v. in PBS. In a pilot study, it was observed that the stated dose of LPS was appropriate to raise body temperature on average by 2°C above the baseline and that it remained elevated for at least 12 hours.

Therapeutic doses of diclofenac (2.5 mg/kg body weight), ketoprofen (3 mg/kg), meloxicam (0.5 mg/kg) and piroxicam (0.5 mg/kg) were given intramuscularly to the animals in the
respective groups at three hours after the injection of LPS while, PBS was given i.m. to the animals in groups 1 and 2.

**Clinical Parameters:**

Rectal temperature (T) in °C was measured by using a clinical thermometer, pulse rate (P) was measured per minute by palpating the mandibular artery, and respiration rate (R) was recorded as the number of inhalations per minute. These observations were recorded for each animal before treatment and then at every hour afterwards, up to 15 hours.

**Blood Collection:**

Blood (3 ml) was collected from each animal through the intravenous catheter in the jugular vein, using K$_3$-EDTA coated Venoject vacutainers with the Quick fit system, Venosafe holders and Venoject needles (Terumo Europe Lab Systems). After the administration of LPS and collection of each blood sample, sterile 3.5% sodium citrate solution was placed in the cannulas to maintain patency and prevent blood clotting. Blood was drawn before the start of experiment, then every three hours up to fifteen hours. After mixing properly, the blood samples were divided into two portions. One ml blood from each sample was immediately used for hematological studies while the remaining sample was centrifuged at 1000 g for 10 min to separate plasma. The plasma was stored at -20° C for biochemical analysis.
Laboratory Parameters:

**Hematology:**

Blood cell counts i.e red blood cell (RBC) count, total leukocyte counts (TLC), differential leukocyte counts (DLC) and platelet counts were performed on the blood samples collected. Hemoglobin (Hb), hematocrit (PCV), mean corpuscle volume (MCV), MCH and MCHC, were also measured in these samples by using a fully automated, Abacus Hematology Analyzer, Micros 60 (Diatron, Austria).

**Biochemical Analyses:**

**Total Protein (TP):**

Total plasma protein was measured by the Biuret method by using Diagnostic Kit, TP 245 (Randox, UK) in which biuret reagent reacts with protein to form a colored compound, the intensity of which is measured by spectrophotometer to estimate the protein concentration.

**Sample:** Serum free of hemolysis.

**Standard:** Bovine serum albumin.

**Principle**

Cupric ions, in an alkaline medium, interact with protein peptide bonds resulting in the formation of a colored complex.

**Reaction Requirements**

<table>
<thead>
<tr>
<th>Requirement</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>546 nm</td>
</tr>
<tr>
<td>Cuvette</td>
<td>1 cm light path</td>
</tr>
<tr>
<td>Incubation temperature</td>
<td>25°C</td>
</tr>
</tbody>
</table>
Measurement against Reagent Blank

The volume of 0.02 ml distilled water, standard solution and each plasma sample were placed in cuvettes labeled as Reagent Blank, Standard and Sample, respectively. Biuret reagent (1 ml) was added and mixed in all three cuvettes and incubated for 30 min at 25°C. The absorbance of sample (A\textsubscript{sample}) and standard (A\textsubscript{standard}) were measured against the reagent blank.

Calculation of Total Protein

Total protein (g/l) = 190X A\textsubscript{sample}/A\textsubscript{standard}X Standard Conc.

Creatine Kinase (CK):

Creatine kinase (CK) activity was measured in plasma by the assay method using creatine phosphate and ADP in a commercially available Kit #1503 (Biocon® Diagnostik, Germany) as described by Oliver (1977) and improved by Szasz (1979). CK is rapidly inactivated by oxidation of the sulfhydryl groups in the active center. The enzyme can be reactivated by the addition of N-acetylcysteine. CK activity is determined by using the “reverse reaction” and activation of enzyme. This assay meets the recommendations of the German Society for Clinical Chemistry and the International Federation of Clinical Chemistry. Additional acronyms used below to describe this assay include: HK: Hexokinase, G6PDH: Glucose-6-phosphate dehydrogenase, NADP: nicotinamide-adenine dinucleotide phosphate, NADPH: Reduced nicotinamide-adenine dinucleotide phosphate.
Test Principle (UV)

Creatine phosphate + ADP $\xrightarrow{\text{CK}}$ Creatine + ATP

ATP + Glucose $\xrightarrow{\text{HK}}$ Glucose-6 P + ADP

Glucose-6-P + NADP $\xrightarrow{\text{G6PDH}}$ Glucose-6-P + NADPH + H^+

Equimolar quantities of NADPH and creatine are formed at the same rate. The photometrically measured rate of formation of NADPH is proportional to the CK activity.

Reaction Requirements

Wavelength 340 nm
Cuvette 1 cm light path
Incubation temperature 37°C
Zero adjustment Distilled water

Procedure

Each plasma sample (20 μl) was mixed with 500 μl of working reagent in a cuvette and incubated for 2 minutes. The increase in the absorbance per minute was measured for 3 minutes.

Calculations

$\text{CK (U/L)} = \Delta A/\text{min} \times 4130.$

Aspartate amino-transaminase (AST) and creatinine (CREA) were estimated in the blood plasma samples using commercial diagnostic kits (Randox, UK) as described in previous experiments. All the photometric tests were conducted using a Spectrophotometer, UV-1650 PC (Shimadzu, Japan).
Statistical Analysis

A completely randomized design was applied to this experiment, and data collected from all the groups for different parameters were analyzed by using two way analysis of variance with the Bonferroni post-Hoc test (p<.0.05) (Neter et al, 1990). All the analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.
EXPERIMENT No. 5

EFFICACY TRIALS OF NONSTEROIDAL ANTIINFLAMMATORY DRUGS IN CLINICALLY AFFECTED ANIMALS UNDER FIELD CONDITIONS

After studying the efficacy of safer alternatives to diclofenac against LPS-induced pyrexia in buffalo calves, the efficacy of the same drugs was evaluated in clinically affected horses. Eighty horses suffering from lameness attributable to hock (tarsal) joint problems and showing variable degrees of inflammation were enrolled for this study.

Inclusion and Exclusion Criteria

The horses (Equuscaballus) were selected on the basis of history, clinical examinations of lameness, pain, swelling and joint mobility after ensuring that the lameness was exclusively attributable to the hock joint. Horses with certain conditions were excluded from this study, including: horses with mechanical lameness, neurologic disorders, septic arthritis, intra-articular fracture, horses treated with alternative therapies or other NSAIDs within 7 days, horses treated with corticosteroids within 30 days, and horses treated with intra-articular injections in the past 90 days or with joint surgery in the past 6 month.

Study Protocol

Before the start of experiment, a complete physical and orthopedic examination of the affected horses was performed. The horses which met the inclusion criteria were randomly allocated to the different treatment groups. The treatment groups, doses and route of administration are shown in Table 3.4.
MATERIALS AND METHODS

Table 3.4: Various NSAIDs used to treat lameness in different groups of horses

<table>
<thead>
<tr>
<th>Group</th>
<th>Drugs used</th>
<th>Dosage</th>
<th>Route of drug administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Meloxicam (MX)</td>
<td>0.5 mg/kg b.w.</td>
<td>I/M</td>
</tr>
<tr>
<td>2.</td>
<td>Piroxicam (PX)</td>
<td>0.5 mg/kg b.w.</td>
<td>&quot;</td>
</tr>
<tr>
<td>3.</td>
<td>Ketoprofen (KP)</td>
<td>1.1 mg/kg b.w.</td>
<td>&quot;</td>
</tr>
<tr>
<td>4.</td>
<td>Normal Saline (NS)</td>
<td>0.5 ml/10kg b.w.</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

The treatment groups MX, PX and KP were treated with meloxicam, piroxicam and ketoprofen, respectively, for four days, while NS group (placebo) was given physiological normal saline solution and served as control. Each horse was evaluated for lameness, pain, inflammation and joint mobility scores using AAEP lameness scale (Table-3.5) before the start of experiment and then daily for five days (Joseph et al., 2002; Keegan et al., 2010). The cumulative scores for an individual animal in each group at all the time points were recorded.

**Blood Sampling**

Venous blood samples were drawn from the jugular vein before the initiation of the experiment and at the end of the observation period. Erythrocyte counts, WBCs and DLCs were performed on these samples.

**Statistical Analysis**

The severity index scores in different treatment groups were compared by two way ANOVA and differenced among the groups at different time points were examined using LSD (p<0.05) (Steel and Torrie, 1982).
Table-3.5: Lameness, Pain, Inflammation and Joint Mobility Scoring System in Horses

<table>
<thead>
<tr>
<th>Lameness Exam*</th>
<th>Joint Pain</th>
</tr>
</thead>
<tbody>
<tr>
<td>0—Lameness not perceptible under any circumstances</td>
<td>0—No pain</td>
</tr>
<tr>
<td>1—Difficult to observe and not consistently apparent</td>
<td>1—Mild pain; horse calmly withdraws limb</td>
</tr>
<tr>
<td>2—Difficult to observe at a walk or when trotting in a straight line, but consistently apparent under certain circumstances</td>
<td>2—Moderate pain; horse withdraws limb and exhibits signs of slight distress</td>
</tr>
<tr>
<td>3—Consistently observable at a trot under all circumstances</td>
<td>3—Severe pain; horse withdraws limb and exhibits signs of severe distress</td>
</tr>
<tr>
<td>4—Obvious at a walk</td>
<td>3—Severe pain; horse draws limb and exhibits signs of severe distress</td>
</tr>
<tr>
<td>5—Minimal weight bearing or inability to move</td>
<td>3—Severe condition</td>
</tr>
</tbody>
</table>

*According to AAEP Lameness Scale

<table>
<thead>
<tr>
<th>Joint Mobility**</th>
<th>Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Compared with day 0)</td>
<td></td>
</tr>
<tr>
<td>0—No change from day 0</td>
<td>0—No swelling</td>
</tr>
<tr>
<td>1—5-10% improvement</td>
<td>1—Mild swelling</td>
</tr>
<tr>
<td>2—11-20% improvement</td>
<td>2—Moderate swelling</td>
</tr>
<tr>
<td>3—&gt;20% improvement</td>
<td>3—Severe swelling</td>
</tr>
</tbody>
</table>

**Initial evaluation on a 0-100% scale, with 100%= normal
EXPERIMENT NO. 1:

DEVELOPMENT OF CHICKEN EXPERIMENTAL MODEL TO STUDY THE TOXICITY OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

In a preliminary study, it was observed that a disease syndrome similar to that reported in vultures after the consumption of diclofenac, can be reproduced in broiler chickens following intramuscular injection of the same drug. Hence the broiler chickens may serve as surrogate model to study the toxicity and safety of at least some NSAIDs and the data may be extrapolated in other avian species including vultures. Therefore this experiment was conducted to evaluate the toxicity of diclofenac in chickens (experimental model) at various dose levels and via the different routes of administration, i.e. oral and intramuscular. One hundred birds were divided into two groups (Group 1 was treated via the intramuscular route and Group 2 was dosed orally). The birds in each group were randomly allocated to five subgroups based on different dose levels that are A (PNS, control); B (diclofenac, 1.25 mg/kg); C (diclofenac, 2.5 mg/kg); D (diclofenac, 5 mg/kg) and E (diclofenac, 10 mg/kg), respectively. The groups treated intramuscularly were denoted as A1, B1, C1, D1 and E1 while the groups exposed via the oral route were designated as A2, B2, C2, D2 and E2, respectively. The following describes the observations of this experiment;

Clinical Findings

Before the start of experiment, all the birds were examined for their physical appearance, behavior and feed and water intake. The birds in all the groups were found clinically healthy. On an average, within 24 hours post-treatment, birds in subgroups, D1, E1, C2, D2, and E2 started showing the signs of toxicity.
The affected birds appeared lethargic, perched isolated from rest of the group. They appeared to be asleep and had stopped eating and drinking. The birds initially responded to external stimuli such as touch and noise and woke up for a while before becoming depressed again. The birds kept their eyes closed for most of the time and had slow but deep breathing. The affected birds were reluctant to move and would only move a few steps when forced and soon sat down again. It was also observed that these birds sit on their knees and seem to have great difficulty while standing and walking (Figure 4.1). The birds that experienced depression, sat on the ground with ruffled feathers, the beak touched the ground, and they were unable to hold their necks in a normal position as the disease progressed. The birds showing severe depression ultimately died, but those that were mildly depressed, survived through the observation period. The birds in the control group remained clinically normal until the end of experiment.

**Mortality**

Mortality rates in the groups treated via the intramuscular route were 0% in A1 and B1 (control and lowest dose chickens) and in groups C1, D1 and E1 mortality was 30%, 40% and 70%, respectively. The groups injected with diclofenac intramuscularly, i.e. A2, B2, C2, D2 and E2 had mortality rates of 0%, 0%, 10%, 30% and 50%, respectively. The majority of birds that died did so from 24 to 36 hours post-exposure. The birds survived of diclofenac toxicity showed no clinical signs throughout the observation period. There was no mortality observed in the control groups (A1, A2). There was a significant difference in mortality as a function of route of drug administration at different dose levels (Tables 4.1, 4.2).
Fig. 4.1: Chicken showing signs of depression after treatment with diclofenac
Table-4.1: Percentage mortality in broiler chickens treated with diclofenac via either the intramuscular (IM) or oral route.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Routes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sub-group</td>
</tr>
<tr>
<td>PNS: (Control)</td>
<td>A1</td>
</tr>
<tr>
<td>Diclofenac sodium (1.25mg/kg)</td>
<td>B1</td>
</tr>
<tr>
<td>Diclofenac sodium (2.5 mg/kg)</td>
<td>C1</td>
</tr>
<tr>
<td>Diclofenac sodium (5 mg/kg)</td>
<td>D1</td>
</tr>
<tr>
<td>Diclofenac sodium (10 mg/kg)</td>
<td>E1</td>
</tr>
</tbody>
</table>

Table-4.2: Survival rates in broiler chickens treated with diclofenac via either the intramuscular (IM) or oral route at different time intervals.

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>IM</th>
<th>Oral</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A1</td>
<td>B1</td>
</tr>
<tr>
<td>0 Hours</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>24 Hours</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>48 Hours</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>72 Hours</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>96 Hours</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Postmortem Examination

Full necropsies were undertaken as soon as possible after the death of birds, and on the birds killed at the end of experiment and the observations were recorded for individual birds. The external features examined for abnormalities included skin, feathers, muscle mass, eyes, nostrils, ears, wings, feet, cloaca and vent. Gross internal observations were made on pectoral muscles, thoracic and abdominal air sacs, heart and major blood vessels, thyroid and adrenal
glands, brain, liver, kidneys, spleen, lungs, trachea, intestinal tract, reproductive organs and tibiotarsal, metatarsal and digital joints. All the birds that died had similar lesions. The major gross lesion was visceral gout manifested as mild to severe and widespread deposition of a mixture of white chalky material (uric acid crystals), white debris (uric acid) and varying amounts of fibrinous exudate on all the visceral organs (Fig. 4.2). A tooth paste-like white material was found in the pericardial sac of affected birds just after death which rapidly solidified to material like powdered sugar when exposed to air. The extent and distribution of gout on internal organs varied among individuals. The precipitation of urates varied from multifocal to locally extensive areas present in the subcutaneous tissues, pectoral muscles, thigh muscles, air sacs, thoracic wall serosal surface, pericardium, epicardium, sternum, abdominal fat, abdominal wall serosal surface, serosal surface of liver, spleen, proventriculus, ventriculus, entire intestinal tract, kidneys, and articular surface within mandibular, hip and hock joints. Small uroliths were found in segments of urethra and urethral openings of the cloaca. The liver was friable and kidneys were pale-tan and swollen. Varied amounts of white debris and fibrin were scattered on most of the visceral serosa. The killed birds showed mild to moderate swelling and pale discoloration of kidneys when examined at the end of experiment. Visceral gout was absent in these birds. No gross lesions were found in the birds of control group (C) and the surviving birds, other than those mentioned above of the treatment groups at the time of necropsy.
Fig 4.2: Visceral gout, a major postmortem lesion in the broiler chicken treated with diclofenac. The urate crystals are deposited on the visceral surfaces of the internal organs (arrows).

**Histopathology**

Birds with visceral gout had deposition of urate material on the visceral surfaces of many organs in association with tissue necrosis when urate precipitation occurred within the parenchyma of organs such as kidney, liver, heart and spleen. Basically, all the dead birds in all treatment groups had such microscopic changes.

**Kidney:**

The kidney sections prepared from birds that died from diclofenac toxicosis had severe renal tubular necrosis with marked urate precipitates. There were multifocal, variably-sized (up to 200 µm in diameter), colorless to basophilic radiating, sharp, acicular, crystalline deposits (urate tophi) that replaced renal tubules and extended into the adjacent cortical interstitium.
These were frequently surrounded by low to moderate numbers of macrophages, fewer multinucleated giant cells, occasional heterophils, and a few areas of scattered cellular and karyorrhectic debris. Multifocally, tubules were mildly dilated (ectatic), with attenuated epithelium, and usually contained a mixture of amorphous eosinophilic material, sloughed epithelial cells, and cellular debris (Fig. 4.4). Tubular epithelial regeneration was not evident in these sections. Most convoluted tubules and collecting tubules had cellular casts. Usually, the glomeruli appeared to be spared. The renal damage was more extensive in the chickens given with higher doses of diclofenac intramuscularly than those dosed orally.

Liver:

There were multifocal, random, variably-sized tophi surrounded by inflammatory cells (multinucleated giant cells and heterophils) in association with hepatocyte necrosis (Fig. 4.6).

Heart:

Multifocal, random, urate tophi as previously described were scattered throughout the cardiac muscle (Fig. 4.8).
Fig 4.3: Histo-micrograph of kidney tissue of untreated broiler chicken. 400X.

Fig 4.4: Histo-micrograph of kidney tissue of broiler chicken treated with diclofenac showing urate crystal deposition (star), damage to the proximal convoluted tubules (thin arrow) infiltration of leukocytes (thick arrow). 400X
EXPERIMENT NO. 1:

Fig 4.5: Histo-micrograph of liver tissue of untreated broiler chicken. 400X

Fig 4.6: Histo-micrograph of liver tissue of broiler chicken treated with diclofenac showing urate. Deposition (star), damaged hepatocytes (arrow head) and infiltration of leukocytes (thin arrows). 400X
EXPERIMENT NO. 1:

Fig 4.7: Histomicrograph of heart tissue of untreated broiler chicken. 400X.

Fig 4.8: Histomicrograph of heart tissue of a broiler chicken showing urate crystal deposition (star) and destruction of cardiac muscles fibers (arrow head) and leukocyte infiltration (thick arrow) after treatment with diclofenac. 400X.
**Skeletal muscle:**

Besides urate tophi, as previously described, there were locally extensive to diffuse areas of acute necrosis with large number of heterophils, some macrophages, a few multinucleated giant cells, cellular and karyorrhectic debris, and severe myodegeneration with intrasarcoplasmic floccular changes accompanied by mild to moderate phagocytosis and satellitosis (Fig. 4.10).

**Spleen:**

The splenic parenchyma contained many scattered urate tophi as previously described (Fig. 4.11).

No histopathological changes were observed in organs of the birds in group A, untreated control (4.3, 4.5, 4.7 and 4.9)

![Histo-micrograph of skeletal muscle of untreated broiler chicken. 400X.](image-url)
Fig 4.10: Histomicrograph of skeletal muscle of the broiler chicken treated with diclofenac shows the deposition of debris of amorphous material (stars), presence of giant cells (arrows) and tissue necrosis (arrow head). 400X.

Fig 4.11: Histomicrograph of broiler chicken spleen tissue showing urate crystal deposition (star) and damage to the parenchyma (arrow) after the administration of diclofenac. 400X.
Biochemical Analyses

Sera collected from broiler chickens of all the groups before the initiation of experiment and 24, 48, 72, and 96 hours after the exposure to diclofenac via the intramuscular (IM) and oral routes were analyzed for uric acid, creatinine, AST, ALT and ALP.

Serum Uric Acid

There were no significant differences among treatment groups in serum uric acid concentrations in samples collected before the start of experiment \((p>0.05)\). Mean ± SD values of serum uric acid concentrations are shown in Tables 4.3 and 4.4 for IM and oral routes, respectively. Uric acid values at 24 and 48 hours post-exposure from subgroups C1, D1 and E1 treated intramuscularly were significantly different from those of subgroup A1 (control). Similarly, subgroups D1 and E1 were significantly different from subgroup A1 at 72 hours. At 24 and 48 hours post-dosing the birds given diclofenac orally of groups C2, D2 and E2 were significant different from the control group (A2) whereas the uric acid concentrations in serum of groups D2 and E2 were significantly greater than controls at 72 hours after the first dose. No significant differences observed between serum uric acid concentrations for each dose for IM and oral routes at all time points except at 2.5 mg/kg at 48 hours post-exposure (Fig. 4.12 & 4.13).
Table-4.3: Serum uric acid concentrations (mg/dL) of broiler chickens treated with different doses of diclofenac via intramuscular (IM) administration (Mean ± SD) as a function of time

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>A1 (control)</th>
<th>B1 (1.25 mg/kg)</th>
<th>C1 (2.5 mg/kg)</th>
<th>D1 (5 mg/kg)</th>
<th>E1 (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td></td>
<td>4.83±0.51</td>
<td>5.26±0.42</td>
<td>4.6±0.33</td>
<td>4.95±0.5</td>
<td>5.08±0.72</td>
</tr>
<tr>
<td>24 hr</td>
<td></td>
<td>4.74±0.96</td>
<td>6.32±1.54</td>
<td>10.38±3.25***</td>
<td>14.2±5.37***</td>
<td>19.62±7.66***</td>
</tr>
<tr>
<td>48 hr</td>
<td></td>
<td>4.92±0.65</td>
<td>6.61±1.37</td>
<td>15.6±6.24***</td>
<td>20.67±8.21***</td>
<td>24.22±5.63***</td>
</tr>
<tr>
<td>72 hr</td>
<td></td>
<td>5.15±0.97</td>
<td>6.72±1.63</td>
<td>6.94±1.03</td>
<td>11.78±1.82***</td>
<td>14.45±2.6***</td>
</tr>
<tr>
<td>96 hr</td>
<td></td>
<td>5.06±0.83</td>
<td>6.58±1.42</td>
<td>6.87±1.56</td>
<td>6.74±1.78</td>
<td>7.32±1.96</td>
</tr>
</tbody>
</table>

*** p<0.001

Table-4.4: Serum uric acid concentrations (mg/dL) of broiler chickens treated with different doses of diclofenac orally (Mean ± SD) as a function of time

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>A2 (control)</th>
<th>B2 (1.25 mg/kg)</th>
<th>C2 (2.5 mg/kg)</th>
<th>D2 (5 mg/kg)</th>
<th>E2 (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td></td>
<td>5.23±0.3</td>
<td>4.86±0.72</td>
<td>4.22±0.4</td>
<td>4.78±0.63</td>
<td>5.62±0.36</td>
</tr>
<tr>
<td>24 hr</td>
<td></td>
<td>4.62±0.25</td>
<td>5.94±1.79</td>
<td>8.67±2.55**</td>
<td>12.05±4.57***</td>
<td>16.81±6.5***</td>
</tr>
<tr>
<td>48 hr</td>
<td></td>
<td>4.94±0.12</td>
<td>6.48±1.68</td>
<td>11.44±4.46***</td>
<td>17.8±6.61***</td>
<td>19.31±5.73***</td>
</tr>
<tr>
<td>72 hr</td>
<td></td>
<td>4.41±0.36</td>
<td>5.73±1.32</td>
<td>6.87±1.36</td>
<td>10.65±1.96***</td>
<td>9.59±3.7***</td>
</tr>
<tr>
<td>96 hr</td>
<td></td>
<td>5.06±0.71</td>
<td>6.36±1.83</td>
<td>6.62±1.25</td>
<td>6.74±2.03</td>
<td>6.84±1.46</td>
</tr>
</tbody>
</table>

** p<0.01
*** p<0.001
EXPERIMENT NO. 1:

Uric Acid IM

Figure 4.12: Graphical representation of mean± SD of serum uric acid concentrations in different groups of broiler chickens treated with diclofenac intramuscularly as a function of time.

Uric Acid Oral

Figure 4.13: Graphical representation of mean ± SD serum uric acid concentrations in different groups of broiler chickens treated with diclofenac orally as a function of time.
Serum Creatinine

Mean ± SD values of serum creatinine concentrations are depicted in Tables 4.5 and 4.6. There was a significant rise in serum creatinine concentrations in samples from birds of group D1 when compared with those in the control group at 48 and 72 hours after the start of experiment. A significant increase was also observed in group E1 at 24, 48 and 72 hours ($p<0.05$). No significant difference from group A1 (control) was recorded in groups B1 and C1 at all the time points.

In group D2, the serum creatinine concentration was significantly higher after 48 hours, and a significant rise was observed for group E2 after 24, 48, and 72 hours post-exposure. No significant difference was seen among groups in serum creatinine concentrations in samples collected at 0 hour (Fig. 4.14, 4.15).
Table-4.5: Serum creatinine concentrations (mg/dL) of broiler chickens treated with different doses of diclofenac via the intramuscular (IM) route (Mean ± SD) as a function of time

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>A1 (control)</th>
<th>B1 (1.25 mg/kg)</th>
<th>C1 (2.5 mg/kg)</th>
<th>D1 (5 mg/kg)</th>
<th>E1 (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td></td>
<td>0.39±0.05</td>
<td>0.37±0.03</td>
<td>0.39±0.07</td>
<td>0.38±0.06</td>
<td>0.37±0.03</td>
</tr>
<tr>
<td>24 hr</td>
<td></td>
<td>0.38±0.04</td>
<td>0.40±0.05</td>
<td>0.52±0.08</td>
<td>0.54±0.16</td>
<td>0.86±0.42**</td>
</tr>
<tr>
<td>48 hr</td>
<td></td>
<td>0.38±0.05</td>
<td>0.42±0.05</td>
<td>0.64±0.32</td>
<td>0.99±0.86***</td>
<td>1.61±0.89***</td>
</tr>
<tr>
<td>72 hr</td>
<td></td>
<td>0.39±0.03</td>
<td>0.41±0.06</td>
<td>0.56±0.18</td>
<td>0.82±0.76*</td>
<td>0.92±0.13†</td>
</tr>
<tr>
<td>96 hr</td>
<td></td>
<td>0.38±0.05</td>
<td>0.40±0.04</td>
<td>0.47±0.11</td>
<td>0.49±0.02</td>
<td>0.52±0.16</td>
</tr>
</tbody>
</table>

*p<0.05
*** p<0.001

Table-4.6: Serum creatinine concentrations (mg/dL) of broiler chickens treated with different doses of diclofenac orally (Mean ± SD) as a function of time.

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>A2 (control)</th>
<th>B2 (1.25 mg/kg)</th>
<th>C2 (2.5 mg/kg)</th>
<th>D2 (5 mg/kg)</th>
<th>E2 (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td></td>
<td>0.38±0.03</td>
<td>0.39±0.05</td>
<td>0.37±0.06</td>
<td>0.38±0.04</td>
<td>0.39±0.05</td>
</tr>
<tr>
<td>24 hr</td>
<td></td>
<td>0.37±0.05</td>
<td>0.42±0.04</td>
<td>0.43±0.04</td>
<td>0.46±0.09</td>
<td>0.76±0.24**</td>
</tr>
<tr>
<td>48 hr</td>
<td></td>
<td>0.39±0.03</td>
<td>0.40±0.07</td>
<td>0.52±0.12</td>
<td>0.78±0.64**</td>
<td>1.14±0.57***</td>
</tr>
<tr>
<td>72 hr</td>
<td></td>
<td>0.38±0.06</td>
<td>0.39±0.05</td>
<td>0.44±0.90</td>
<td>0.70±0.43</td>
<td>0.80±0.23**</td>
</tr>
<tr>
<td>96 hr</td>
<td></td>
<td>0.37±0.03</td>
<td>0.43±0.10</td>
<td>0.43±0.07</td>
<td>0.49±0.09</td>
<td>0.53±0.13</td>
</tr>
</tbody>
</table>

*p<0.05
** p<0.01
*** p<0.001
EXPERIMENT NO. 1:

Creatinine IM

![Creatinine IM graph]

Figure 4.14: Graphs showing mean ± SD serum creatinine concentrations in different groups of broiler chickens treated with diclofenac intramuscularly as a function of time.

Creatinine Oral

![Creatinine Oral graph]

Figure 4.15: Graphs showing mean ± SD serum creatinine concentrations in different groups of broiler chickens treated with diclofenac orally as a function of time.
**Serum AST**

A significant increase in serum AST activity was observed in samples from the birds of groups C1, D1 and E1 at 24, 48 and 72 hours after dosing \((p<0.05)\). A similar rise was recorded in all the treatment groups compared to the control group at 96 hours (Fig. 4.16, 4.17).

Groups C2 and D2 showed a significant rise in serum AST activity at 24 and 48 hours. The enzyme activity in group E2 was significantly higher at all the time points \((p<0.05)\).

There observed no significant difference in the enzyme activity among all the groups at the start of experiment. Mean ± SD values of serum AST are shown in Tables 4.7 and 4.8.
Table-4.7: Serum AST activities (U/L) of broiler chickens treated with different doses of diclofenac via the intramuscular route (Mean ± SD) as a function of time

<table>
<thead>
<tr>
<th>Group</th>
<th>A1 (control)</th>
<th>B1 (1.25 mg/kg)</th>
<th>C1 (2.5 mg/kg)</th>
<th>D1 (5 mg/kg)</th>
<th>E1 (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td>126.79±14.95</td>
<td>123.99±13.57</td>
<td>125.12±18.04</td>
<td>125.01±17.11</td>
<td>127.62±16.12</td>
</tr>
<tr>
<td>24 hr</td>
<td>121.07±11.15</td>
<td>137.06±15.66</td>
<td>171.18±35.10   **</td>
<td>192.95±43.30***</td>
<td>232.38±48.44***</td>
</tr>
<tr>
<td>48 hr</td>
<td>126.21±13.77</td>
<td>146.16±17.65</td>
<td>197.29±51.92***</td>
<td>227.97±60.45***</td>
<td>250.98±70.03***</td>
</tr>
<tr>
<td>72 hr</td>
<td>126.10±15.07</td>
<td>155.42±19.90</td>
<td>168.90±42.52   *</td>
<td>184.11±34.44**</td>
<td>204.55±37.40***</td>
</tr>
<tr>
<td>96 hr</td>
<td>123.80±16.81</td>
<td>161.98±16.10    *</td>
<td>163.17±24.55*</td>
<td>175.86±29.12**</td>
<td>190.71±29.98**</td>
</tr>
</tbody>
</table>

* p<0.05  
** p<0.01  
*** p<0.001

Table-4.8: Serum AST activities (U/L) of broiler chickens treated with different doses of diclofenac via the oral route (Mean ± SD) as a function of time

<table>
<thead>
<tr>
<th>Group</th>
<th>A2 (control)</th>
<th>B2 (1.25 mg/kg)</th>
<th>C2 (2.5 mg/kg)</th>
<th>D2 (5 mg/kg)</th>
<th>E2 (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td>123.52±19.48</td>
<td>129.31±18.83</td>
<td>131.53±12.44</td>
<td>120.72±15.86</td>
<td>131.21±17.23</td>
</tr>
<tr>
<td>24 hr</td>
<td>127.26±13.58</td>
<td>130.06±19.20</td>
<td>162.83±33.64*  **</td>
<td>174.26±28.12***</td>
<td>206.78±39.64***</td>
</tr>
<tr>
<td>48 hr</td>
<td>120.62±16.37</td>
<td>139.74±25.28</td>
<td>183.64±42.60***</td>
<td>197.64±43.86***</td>
<td>242.35±52.75***</td>
</tr>
<tr>
<td>72 hr</td>
<td>129.47±14.72</td>
<td>142.63±19.11</td>
<td>156.31±25.69</td>
<td>159.67±36.75</td>
<td>189.85±33.80***</td>
</tr>
<tr>
<td>96 hr</td>
<td>122.93±15.60</td>
<td>154.63±11.84*</td>
<td>143.58±27.97</td>
<td>154.02±27.32</td>
<td>172.24±26.79**</td>
</tr>
</tbody>
</table>

* p<0.05  
** p<0.01  
*** p<0.001
Figure 4.16: Graphs showing mean ± SD serum AST activities in different groups of broiler chickens treated with diclofenac intramuscularly as a function of time.

Figure 4.17: Graphs showing mean ± SD serum AST activities in different groups of broiler chickens treated with diclofenac orally.
Serum ALT

No significant difference was evident in serum ALT activities among the groups prior to initiation of the experiment. Mean ± SD values of serum ALT activities are presented in Tables 4.9 and 4.10. In groups C1, D1 and E1, there was a significant rise in the enzyme activities at all the post-dosing sampling times when compared to t=0 (p<0.05). A similar pattern of rise in the ALT activity was manifested in groups C2, D2 at 24, 48, 72 and 96 hours after first dosing of the diclofenac (Fig. 4.18, 4.19).
**Table-4.9: Serum ALT Activities (U/L) of broiler chickens treated with different doses of diclofenac via the intramuscular route (Mean ± SD) as a function of time**

<table>
<thead>
<tr>
<th>Group</th>
<th>A1 (control)</th>
<th>B1 (1.25 mg/kg)</th>
<th>C1 (2.5 mg/kg)</th>
<th>D1 (5 mg/kg)</th>
<th>E1 (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td>23.06±3.24</td>
<td>23.43±3.53</td>
<td>24.34±2.62</td>
<td>24.71±3.15</td>
<td>22.82±4.96</td>
</tr>
<tr>
<td>24 hr</td>
<td>22.26±3.46</td>
<td>24.41±4.02</td>
<td>33.32±5.05***</td>
<td>54.58±7.24***</td>
<td>63.33±6.2***</td>
</tr>
<tr>
<td>48 hr</td>
<td>21.61±5.84</td>
<td>26.1±3.87</td>
<td>56.79±7.44***</td>
<td>66.17±6.7***</td>
<td>71.07±9.09***</td>
</tr>
<tr>
<td>72 hr</td>
<td>25.15±4.91</td>
<td>29.87±3.32</td>
<td>45.18±5***</td>
<td>54.78±8.53***</td>
<td>58.88±8.42***</td>
</tr>
<tr>
<td>96 hr</td>
<td>23.84±3.23</td>
<td>32.72±2.25***</td>
<td>36.63±6.75***</td>
<td>42.36±6.73***</td>
<td>47.52±6.94***</td>
</tr>
</tbody>
</table>

*** p<0.001

**Table-4.10: Serum ALT activities (U/L) of broiler chickens treated orally with different doses of diclofenac (Mean ± SD) as a function of time**

<table>
<thead>
<tr>
<th>Group</th>
<th>A2 (control)</th>
<th>B2 (1.25 mg/kg)</th>
<th>C2 (2.5 mg/kg)</th>
<th>D2 (5 mg/kg)</th>
<th>E2 (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td>23.18±5.07</td>
<td>22.7±5.75</td>
<td>25.16±4.36</td>
<td>24.12±4.43</td>
<td>21.64±4.33</td>
</tr>
<tr>
<td>24 hr</td>
<td>21.79±8.4</td>
<td>23.11±4.86</td>
<td>29.53±4.67*</td>
<td>48.25±7.78***</td>
<td>56.74±6.86***</td>
</tr>
<tr>
<td>48 hr</td>
<td>24.21±7.37</td>
<td>25.08±6.26</td>
<td>43.31±5.24***</td>
<td>58.85±8.32***</td>
<td>64.45±8.43***</td>
</tr>
<tr>
<td>72 hr</td>
<td>22.85±4.19</td>
<td>26.14±4.75</td>
<td>37.8±6.75***</td>
<td>46.67±6.24***</td>
<td>51.23±7.22***</td>
</tr>
<tr>
<td>96 hr</td>
<td>21.59±6.87</td>
<td>28.47±7.06</td>
<td>30.29±4.28*</td>
<td>37.25±5.63***</td>
<td>42.14±5.56***</td>
</tr>
</tbody>
</table>

* p<0.05  
*** p<0.001
Figure 4.18: Mean ± SD serum ALT activities shown graphically of different groups of broiler chickens treated intramuscularly with diclofenac as a function of time.

Figure 4.19: Mean ± SD serum ALT activities shown graphically in different groups of broiler chickens treated orally with diclofenac as a function of time.
Serum ALP

Serum ALP activities increased significantly in groups C1, D1, E1, C2, D2 and E2 after 24 and 48 hours (p<0.05). In groups B1 and B2, there was no significant rise in enzyme activities as compared to groups A1 and A2, respectively (Fig. 4.20, 4.21). No significant difference was observed among groups in pre-dosing serum ALP activities.

Mean±SD values of serum ALP are shown in Tables 4.11 and 4.12 for the IM and oral routes, respectively.
### Table-4.11: Serum ALP activities (U/L) of broiler chickens treated with different doses of diclofenac via the intramuscular route (Mean ± SD) as a function of time

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>A1 (control)</th>
<th>B1 (1.25 mg/kg)</th>
<th>C1 (2.5 mg/kg)</th>
<th>D1 (5 mg/kg)</th>
<th>E1 (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr</td>
<td>525.8±43.02</td>
<td>530.15±29.87</td>
<td>524.05±46.33</td>
<td>532.54±37.18</td>
<td>526.17±41.96</td>
</tr>
<tr>
<td></td>
<td>24 hr</td>
<td>523.44±38.12</td>
<td>545.41±40.06</td>
<td>597.13±48.44***</td>
<td>646.35±44.56***</td>
<td>658.79±40.52***</td>
</tr>
<tr>
<td></td>
<td>48 hr</td>
<td>526.88±44.1</td>
<td>551.64±45.81</td>
<td>632.06±38.91***</td>
<td>685.58±67.68***</td>
<td>691.82±67.06***</td>
</tr>
<tr>
<td></td>
<td>72 hr</td>
<td>527.93±38.7</td>
<td>550.16±37.12</td>
<td>577.34±43.1</td>
<td>550.11±44.01</td>
<td>581.31±46.77</td>
</tr>
<tr>
<td></td>
<td>96 hr</td>
<td>530.42±29.65</td>
<td>549.62±40.1</td>
<td>554.1±40.45</td>
<td>547.63±32.9</td>
<td>553.05±30.78</td>
</tr>
</tbody>
</table>

*** p<0.001

### Table-4.12: Serum ALP activities (U/L) in broiler chickens treated orally with different doses of diclofenac (Mean ± SD) as a function of time

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>A2 (control)</th>
<th>B2 (1.25 mg/kg)</th>
<th>C2 (2.5 mg/kg)</th>
<th>D2 (5 mg/kg)</th>
<th>E2 (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr</td>
<td>532.21±43.28</td>
<td>527.47±34.9</td>
<td>528.52±28.53</td>
<td>522.12±32.54</td>
<td>531.82±38.03</td>
</tr>
<tr>
<td></td>
<td>24 hr</td>
<td>524.47±23.31</td>
<td>541.2±48.51</td>
<td>579.37±43.9*</td>
<td>610.73±42.91***</td>
<td>634.27±49.23***</td>
</tr>
<tr>
<td></td>
<td>48 hr</td>
<td>529.05±49.86</td>
<td>544.37±37.42</td>
<td>606.42±47.39***</td>
<td>642.7±34.62***</td>
<td>653.55±54.96***</td>
</tr>
<tr>
<td></td>
<td>72 hr</td>
<td>521.53±39.69</td>
<td>549.69±40.76</td>
<td>558.25±36.75</td>
<td>547.34±28.94</td>
<td>566.2±32.05</td>
</tr>
<tr>
<td></td>
<td>96 hr</td>
<td>534.07±48.24</td>
<td>542.5±44.1</td>
<td>551.34±33.85</td>
<td>546.08±29.78</td>
<td>546.67±35.08</td>
</tr>
</tbody>
</table>

* p<0.05  
** p<0.01  
*** p<0.001
Figure 4.20: Graphical representation of mean ± SD serum ALP activities in different groups of broiler chickens treated with diclofenac via the intramuscular route as a function of time.

Figure 4.21: Graphical representation of Mean ± SD serum ALP activities in different groups of broiler chickens treated orally with diclofenac as a function of time.
EXPERIMENT NO. 2

COMPARATIVE TOXICITY OF DICLOFENAC IN DIFFERENT AVIAN SPECIES

The objective of this experiment was to compare the toxicity of diclofenac in different avian species, i.e. chickens, pigeons and quails. Each group of birds was treated intramuscularly with toxic doses of diclofenac (10mg/kg) intramuscularly for four consecutive days. Study parameters included serum biochemical values, clinical signs, mortality, and gross and microscopic lesions.

Clinical Signs

Chickens and pigeons in the treatment groups started showing signs of toxicosis approximately thirty-six hours post-exposure. The clinical signs shown by these two species were similar, and included moderate to severe depression, lethargy, anorexia, and reluctance to move. Affected birds appeared unable to stand and, if they were forced to stand and move, they moved only for a very brief period of time and sat down again. Most of the diclofenac-treated birds sat on their hocks. At advanced stages, the affected birds did not hold their necks in the normal position, and they rested, lying on the ground with eyes closed and the beak touching the ground. The respiration of the affected birds was deep and slow. At the terminal stages, the affected birds became comatose and were found dead in the same posture. No signs of toxicosis were evident in quails given diclofenac. The chickens, pigeons, and quails in the control groups remained healthy and showed no clinical signs throughout the observation period.
**Mortality**

Mortality due to diclofenac toxicosis occurred in treated groups for the chickens and pigeons. Mostly, these birds died from 48 to 72 hours after administration of first dose. A total of seven diclofenac-treated chickens died. Of these, five died between 48 and 72 hours and the remaining two died between 72 and 96 hours. In pigeons given diclofenac, three died between 48 and 72 hours, and one died after 72 hours post-exposure. All quails exposed to the diclofenac survived. None of the control birds died.

**Postmortem Examination**

The chickens and pigeons that died from diclofenac toxicosis exhibited similar lesions although they were more severe in the former. The main gross lesion was visceral gout. A white chalky material was deposited on the visceral surfaces of the internal organs and tissues, including liver, heart, lungs, kidneys, gastrointestinal tract, air sacs, skeletal muscle surface and sub-cutis (Fig. 4.22, 4.23). Such deposits were also observed in all major joints including the hocks, knees, and temporomandibular. Other lesions included swelling of the kidneys, and a mottling and a friable texture to the liver. The chickens and pigeons that survived, and all quails were killed at the end of observation period. Three chickens and one pigeon (all in diclofenac treatment groups) showed mild to moderate swelling of kidneys. One diclofenac-dosed chicken that survived and the pigeons given the drug had mild visceral gout. No postmortem lesions were observed in the diclofenac-treated quail group and the birds in the control groups (Fig 4.24).
Figure 4.22: A carcass of a broiler chicken treated with diclofenac shows massive deposition of urate crystals on the surfaces of visceral organs (arrows).

Figure 4.23: A carcass of a pigeon treated with diclofenac. Urate crystal deposition is shown on visceral organs like heart, liver, lungs and muscles (arrows).
Figure 4.24: A carcass of a quail treated with diclofenac. No urate crystal deposition is evident.

**Histopathology**

The microscopic lesions in chickens that displayed signs of diclofenac toxicosis were identical to those observed in experiment No. 1 (Figs. 4.25, 4.28, 4.31, 4.34). In pigeons that died from diclofenac toxicosis microscopic lesions were evident in almost all the tissues collected. Mild to extensive tubular necrosis in areas of aggregation of urate crystals was present in birds affected by diclofenac toxicosis. Urate tophi were distributed mainly in the cortical region and the medulla was less affected. The normal architecture of the kidney was disrupted in the areas of urate deposition. Urate tophi were surrounded by multinucleated giant cells. There was macrophage and heterophil infiltration in interstitial spaces. In addition to the tophi, there was a deposition of amorphous material inside tubular lumens and collecting ducts (Figs. 4.26, 4.29, 4.32, 4.35). In quails no abnormal microscopic lesions were recorded in all the treated birds (Figs. 4.27, 4.30, 4.33, 4.36).
Figure 4.25: Histomicrograph of kidney of a chicken treated with diclofenac. Urate deposition (star) with damage to proximal tubules (thin arrow) and leukocyte infiltration (thick arrow) are prominent. 200X.

Figure 4.26: Histomicrograph of kidney of pigeon treated with diclofenac showing urate deposition (star), leukocyte infiltration (thick arrow) and tubular damage (thin arrow). 200X.
Figure 4.27: Histomicrograph of the kidney of a quail treated with diclofenac. Normal proximal tubules (thin arrow) and glomeruli (thick arrow) are visible. 100X.

Figure 4.28: Histomicrograph of liver of a chicken treated with diclofenac. Urate deposition (star), leukocytic infiltration (thick arrow), and hepatocyte damage (thin arrow) are evident. 200X.
Figure 4.29: Histomicrograph of liver tissue of a pigeon treated with diclofenac. Massive leukocytic infiltration (thick arrow) and hepatocyte damage (thin arrow) are evident. Silhouettes of urate deposition on hepatic capsule are also seen (star). 200X.

Figure 4.30: Histomicrograph of liver tissue of quail treated with diclofenac. Hepatocytes are normal (arrow). 200X.
Figure 4.31: Histomicrograph of heart muscle of a chicken treated with diclofenac. Urate crystal deposition (star) with leukocytic infiltration (thick arrow) has damaged the muscle fibers (thin arrow). 200X.

Figure 4.32: Histomicrograph of myocardium of a pigeon treated with diclofenac. Urate crystal deposition (star) with leukocytic infiltration (thin arrow) damaged the muscle fibers (thick arrow). Urate silhouettes (arrow head) on the muscle surface are seen. 200X.
Figure 4.33: Histomicrograph of myocardium of quail treated with diclofenac. No urate deposition seen. The muscle tissue is normal. 200X.

Figure 4.34: Histomicrograph of skeletal muscle of a chicken treated with diclofenac. Urate crystal deposition (star) with heavy leukocyte infiltration (thick arrow) has damaged the muscle fibers (thin arrow). 400X. Muscle cell necrosis is also evident.
Figure 4.35: Histomicrograph of skeletal muscle of a pigeon treated with diclofenac. Urate crystal deposition (star) on the muscle surface with mild leukocyte infiltration (thick arrow). Muscle cell necrosis is also present (thin arrow). 400X.

Figure 4.36: Histomicrograph of skeletal muscle of a quail treated with diclofenac. Urate crystal deposition is not present. Normal muscle fibers are seen. 200X.
Serum Biochemical Analyses

The normal values of uric acid, creatinine concentrations, and AST, ALT and ALP activities varied among different species, thus, it was not possible to compare these values as such. Therefore, increases in these values in response to diclofenac in the treatment groups were, first converted into percent increase from the baseline values, and the percent increases in values at 24 and 48 hours after the start of experiment were compared among species.

Uric Acid

The mean±SD values of serum uric acid concentrations for chickens, pigeons and quail before the beginning of the experiment and 24 and 48 hours after first the exposure are given in Table-4.13. There was a significant increase in uric acid concentrations at 24 hours and 48 hours post-treatment compared with baseline values in chickens, pigeons and quail (p<0.05). No significant difference was observed for these values in the control groups of these species, values of control groups of chickens, pigeons and quail had serum uric acid within the normal range throughout the observation period.

The percentage increases in uric acid concentrations from baseline values were calculated and compared for all three species (Fig. 4.37). There was a significant difference in percent increase in uric acid concentrations among the treatment groups of chickens, pigeons and quail.
Table 4.13: Serum uric acid concentrations (mg/dL) in different bird species treated with diclofenac at different time intervals (Mean±SD)

<table>
<thead>
<tr>
<th>Species</th>
<th>Chicken</th>
<th>Pigeon</th>
<th>Quail</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>Control</td>
</tr>
<tr>
<td>Time</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>0 Hour</td>
<td>4.85±0.41</td>
<td>4.82±0.32</td>
<td>5.54±0.53</td>
</tr>
<tr>
<td>24 Hour</td>
<td>4.84±0.28</td>
<td>11.16±3.27a</td>
<td>5.54±0.55</td>
</tr>
<tr>
<td>48 Hour</td>
<td>4.82±0.40</td>
<td>16.33±5.9a</td>
<td>5.52±0.54</td>
</tr>
</tbody>
</table>

Values with different superscripts differ significantly.

Figure 4.37: Graph showing percent increase in serum uric acid concentrations (Mean±SD) in different bird species after treatment with diclofenac.
Creatinine

Mean±SD values of serum creatinine for chickens, pigeons and quails in control and treatment groups at different time intervals are shown in Table-4.14. There was a significant rise in serum creatinine concentration in the samples collected at 24 and 48 hours from chicken and pigeon groups treated with diclofenac when compared with the control group. The serum creatinine values did not vary significantly between treatment and control groups of quail (p<0.05). The percentage increase in the serum creatinine concentration varied significantly among chickens, pigeons and quails (Fig. 4.38).
Table-4.14: Serum creatinine concentrations (mg/dL) in different bird species treated with diclofenac at different time intervals (Mean±SD)

<table>
<thead>
<tr>
<th>Species</th>
<th>Chicken</th>
<th>Pigeon</th>
<th>Quail</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>Control</td>
</tr>
<tr>
<td>Time</td>
<td>Mean± SD</td>
<td>Mean± SD</td>
<td>Mean± SD</td>
</tr>
<tr>
<td>0 Hour</td>
<td>0.42± 0.02</td>
<td>0.42± 0.04</td>
<td>0.81± 0.04</td>
</tr>
<tr>
<td>24 Hour</td>
<td>0.4± 0.04</td>
<td>0.67± 0.19a</td>
<td>0.82± 0.03</td>
</tr>
<tr>
<td>48 Hour</td>
<td>0.42± 0.04</td>
<td>0.99± 0.39a</td>
<td>0.81± 0.03</td>
</tr>
</tbody>
</table>

Values with different superscripts differ significantly

Figure 4.38: Percent increase in serum creatinine (Mean±SD) in different bird species after treatment with diclofenac
Serum AST

There was a great variation among the normal values of serum AST for chickens, pigeons and quail. However, no significant difference was present in these values between treatment and control groups of individual species at 0 hour. Mean±SD serum AST activities in control and treatment groups of chickens, pigeons and quail are shown in Table-4.15. Increases in AST activities were observed in chickens and pigeons. A significant rise was recorded in the values of AST at 24 and 48 hours after the first exposure in treatment groups of chicken and pigeon when compared with the baseline values \( p<0.05 \). The percent increases in serum AST activities at 24 and 48 hours after the start of the experiment comparing chickens, pigeons and quail are shown in Fig 4.39. There were significant differences in the percentage increase of serum AST activities among the three species. The enzyme activities in all control groups remained in the normal range throughout the observation period.
Table 4.15: Serum aspartate transaminase activities (U/L) in different bird species treated with diclofenac at different time intervals (Mean±SD)

<table>
<thead>
<tr>
<th>Species</th>
<th>Chicken</th>
<th>Pigeon</th>
<th>Quail</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>Control</td>
</tr>
<tr>
<td>Time</td>
<td>Mean±</td>
<td>Mean±</td>
<td>Mean±</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
</tr>
<tr>
<td>0 Hour</td>
<td>128.23±8.87</td>
<td>128.6±8.8</td>
<td>133.64±8.36</td>
</tr>
<tr>
<td>24 Hour</td>
<td>127.14±8.32</td>
<td>171.75±26.38 a</td>
<td>139.03±7.37</td>
</tr>
<tr>
<td>48 Hour</td>
<td>132.23±7.58</td>
<td>226.1±42 a</td>
<td>142.26±6.72</td>
</tr>
</tbody>
</table>

Values with different superscripts differ significantly

Figure 4.39: Percent increase in serum aspartate transferase activities (Mean±SD) in different bird species after treatment with diclofenac.
Serum ALT

Mean±SD values of serum ALT at 0, 24 and 48 hours for control and treatment groups of chickens, pigeons and quails are presented in Table-4.16. Serum ALT activities of treatment and control groups in each species were not significantly different before the start of experiment. In chickens and pigeons, the activities of this enzyme were significantly higher in the treatment groups at 24 and 48 hours after the initial treatment compared to control groups (Fig 4.40). The enzyme activities of all control groups of three species remained within normal limits throughout the experiment.
Table 4.16: Serum alanine transaminase activities (U/L) in different bird species treated with diclofenac at different time intervals (Mean±SD)

<table>
<thead>
<tr>
<th>Species</th>
<th>Chicken</th>
<th>Pigeon</th>
<th>Quail</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>Control</td>
</tr>
<tr>
<td><strong>Time</strong></td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>0 Hour</td>
<td>22.7±2.45</td>
<td>21.86±2.57</td>
<td>15.16±1.62</td>
</tr>
<tr>
<td>24 Hour</td>
<td>22.65±2.97</td>
<td>36.68±7.25</td>
<td>15.38±1.44</td>
</tr>
<tr>
<td>48 Hour</td>
<td>23.06±2.83</td>
<td>55.08±14.65a</td>
<td>15.47±1.33</td>
</tr>
</tbody>
</table>

Values with different superscripts differ significantly

Serum alanine transaminase activities

![Figure 4.40: Percent increase in serum ALT activities (Mean±SD) in different bird species after treatment with diclofenac](image)

Figure 4.40: Percent increase in serum ALT activities (Mean±SD) in different bird species after treatment with diclofenac
Serum ALP

Serum ALP values were quite different among the three avian species (Table-4.17). Before the start of the treatment period the activities of this enzyme for chickens, pigeons, and quail were not significantly different between the control and treatment groups. A significant increase in ALP activities was observed in groups of the chickens and pigeons given diclofenac as compared to their respective control groups at 24 and 48 hours after diclofenac administration (p<0.05). Significant increases in percent serum ALP activities were recorded in the chickens and pigeons when compared to the quail treatment groups (Fig. 4.41).
Table 4.17: Serum ALP activities (U/L) in different bird species treated with diclofenac at different time intervals (Mean±SD)

<table>
<thead>
<tr>
<th>Species</th>
<th>Chicken</th>
<th>Pigeon</th>
<th>Quail</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>Treated</td>
<td>Control</td>
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<tr>
<td>Time</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>0 Hour</td>
<td>546.91±23.58</td>
<td>551.44±29.01</td>
<td>354.52±20</td>
</tr>
<tr>
<td>24 Hour</td>
<td>552.33±19.36</td>
<td>606.89±38.32</td>
<td>363.03±21.89</td>
</tr>
<tr>
<td>48 Hour</td>
<td>567.49±24.28</td>
<td>661.04±38.32</td>
<td>371.24±30.79</td>
</tr>
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</table>

Values with different superscripts differ significantly.

Serum alkaline phosphatase activities

![Graph showing percent increase in serum alkaline phosphatase activities for different bird species after treatment with diclofenac]

Figure 4.41: Percent increase in serum alkaline phosphatase activities (Mean±SD) in different bird species after treatment with diclofenac
Summary

In this experiment, visceral gout developed in the chickens and pigeons treated with diclofenac sodium at the dose of 10 mg/kg body weight, while no lesions of this syndrome were present in quail treated with the same dose of diclofenac.

Significant increases in serum values of uric acid, creatinine, AST, ALT and ALP were observed in chickens and pigeons, but the values for these parameters remained normal in quail. The morphology and physiology of various organs also changed in the affected birds.

It is concluded that a great variation exists among different bird species regarding the sensitivity to the diclofenac toxicity. Broiler chickens are more sensitive than pigeons and quail. The quail are more resistant to the toxic effects of diclofenac than chickens and pigeons.
On completion of the first and second experiments, it was concluded that diclofenac was toxic to the broiler chickens, pigeons and, to a lesser degree, quail. Of course the need has arisen to find an alternative to diclofenac, which should be both efficacious in target domestic animal species and comparatively safer for scavenger species, especially birds.

In this experiment, six NSAIDs were evaluated for their toxicity profile and compared with that of diclofenac in broiler chickens (Experimental Model). These compounds included phenylbutazone (FBZ), metamizole (MTZ), meloxicam (MX), piroxicam (PX), flunixin meglumine (FM) and ketoprofen (KP) and diclofenac (DF). These NSAIDs are being used in human and veterinary medicine.

During previous experiments it was observed that, although toxic effects and mortality occurred in the broiler chickens even when exposed intramuscularly to the therapeutic doses of diclofenac, i.e. 2.5 mg/kg body weight, these effects were more severe at 10 mg/kg. Therefore, it was decided to evaluate the various NSAIDs at four times the normal doses.

The following observations were recorded during the course of the third experiment.

Clinical Findings

Birds in the NSAID treatment and control groups were examined on commencement of the experiment and found to be clinically healthy. Clinical signs were manifested by the birds of the FM and DF groups, while no significant clinical observations were recorded in the other treatment groups. The clinical signs recorded in the birds of these groups were identical to those observed in the first experiment with DF. All of the affected birds in the FM and DF
groups exhibited variable degrees of depression characterized by reluctance to move, tendency to sit down most of the time, and slow, deep respiration.

These birds remained sitting with closed eyes and head down so that the beak touched to the ground. When the birds were forced to move, they moved only a few steps and then sat down.

Most affected birds sat on their hocks and some of them walked on their hocks (Fig 4.42). The birds were anorexic and seldom ate or drank after the onset of the aforementioned clinical signs. The feathers of the affected chickens were ruffled. The affected birds started showing signs of toxicity approximately 24 hours after the first dose of FM or DF. Severely affected birds remained alive for 24 to 48 hours after appearance of the first signs of toxicity. The birds showing severe degree of depression ultimately died, but those that became only mildly depressed survived throughout the observation period. The birds in the control group remained clinically normal until the end of experiment.

Prior to death, the affected birds became progressively more depressed, appeared to be comatose, and could not be aroused. The birds were often found dead. No clinical signs of NSAID toxicosis were observed in birds of the other treatment and control groups, and they remained clinically healthy throughout the observation period.
Mortality

The only birds that died were in the FM and DF groups. Most deaths occurred from 48 to 72 hours after the first dosing of the drugs. Sixty and seventy percent mortality rates were recorded for the FM and DF groups, respectively. The mortality pattern for FM and DF is shown in Table-4.18. The birds in the control group remained normal throughout the experiment.
Table-4.18: Mortality patterns in different groups of broiler chicken treated with different NSAIDs

<table>
<thead>
<tr>
<th>Time after initial dose (Hours)</th>
<th>FZB</th>
<th>MTZ</th>
<th>MX</th>
<th>PX</th>
<th>KP</th>
<th>FM</th>
<th>DF</th>
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<td>7</td>
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</tbody>
</table>

FZB = phenylbutazone, MTZ = metamizole, MX = meloxicam, PX = piroxicam, KP = ketoprofen, FM = flunixin meglumine, DF = diclofenac

**Postmortem Examination**

All the birds that died in the FM and DF groups had similar lesions. The major gross lesion was visceral gout manifested as mild to severe and widespread deposition of a mixture of white chalky material (uric acid crystals), white debris (uric acid) with a varied amount of fibrinous exudates on all the visceral organs (Fig. 4.43). The extent and distribution of uric acid crystals on internal organs varied among individuals. The precipitation of urates varied from multifocal to locally extensive areas present in the subcutaneous tissues, pectoral muscles, thigh muscles, air sacs, thoracic wall serosal surface, pericardium, epicardium, sternum, abdominal fat, abdominal wall serosal surface, serosal surface of liver, spleen, proventriculus, ventriculus, entire intestinal tract, kidneys, and articular surface within the
temporo-mandiblar, hip and hock joints. Small uroliths were found in segments of the urethra and the urethral opening into the cloaca.

The liver was friable and kidneys were pale-tan and swollen. Varied amounts of white debris and fibrin were scattered on most of the visceral serosa.

Minimal to severe necrosis was observed at the site of injection in the pectoral muscle in chickens of the MTZ, FM, and FBZ groups. These types of lesions were not present in the other groups. In the groups given PBZ and MTZ focal to diffuse hepatic necrosis was evident in 6 and 4 birds, respectively. In the groups given MX, PX and KP, mild focal hepatic discoloration was evident in few birds. No gross lesions were recorded in the birds of the control groups.
Histopathology

The birds in groups given FM and DF that exhibited clinical signs of toxicity and subsequently died showed postmortem lesions of visceral gout, with urate deposition on and inside the visceral organs. The urate deposition was associated with tissue necrosis in the kidney, liver, heart, and spleen.

**Kidney:**

Kidney sections of the birds given DF and FM had lesions very similar to those recorded in the first experiment, including severe renal tubular necrosis with marked urate precipitation. Multifocal, variably-sized (up to 200 µm in diameter) crystalline deposits (urate tophi) were found in the histologic kidney sections. These tophi were colorless to basophilic, radiating, sharp, and acicular in appearance. The deposits had expanded and replaced the renal tubules. The crystals extended into the adjacent cortical interstitium. The urate tophi were frequently
surrounded by low to moderate numbers of macrophages, a fewer multinucleated giant cells, occasional heterophils, and a limited amount of scattered cellular and karyorrhectic debris. Multifocally, tubules were mildly dilated (ectatic), with attenuated epithelium. Most such tubules contained a mixture of amorphous eosinophilic material, sloughed epithelial cells and cellular debris (Fig. 4.44). Tubular epithelial regeneration was not evident in these sections. Most convoluted tubules and collecting ducts had cellular casts. Usually the glomeruli appeared to be spared. The renal damage caused by FM was comparable to that observed with DF. Kidney from birds of the other treatment groups and from those of the control group showed no significant histopathologic changes.

Figure 4.44: Histomicrograph of kidney of broiler chickens treated with flunixin meglumine showing urate crystal deposition (star). Tubular necrosis (thin arrow) with leukocyte infiltration (thick arrow) is evident. 400X.

Liver:
There were multifocal, random, variably-sized tophi surrounded by inflammatory cells (multinucleated giant cells and heterophils) associated with areas of hepatocyte necrosis in affected birds of the groups given DF and FM (Fig. 4.45). Hepatic necrotic areas were present in the groups given MX, PX, KP, MTZ and PBZ (Fig 4.48 to 4.50). Urate crystal deposition was not observed in the chickens of these groups. The liver tissues processed from the control group showed no abnormalities.

**Heart:**

In groups given DF and FM, lesions included multifocal, random, urate tophi scattered throughout the cardiac muscle (Fig. 4.46). The cardiac tissues of the birds from other treatment groups and the control group showed no lesions.

**Skeletal muscle:**

In addition to the urate tophi, locally extensive to diffused areas of acute necrosis with large number of heterophils, some macrophages, a few multinucleated giant cells, and cellular and karyorrhectic debris were found in the birds given DF and FM. Severe myodegeneration, with intra-sarcoplasmic floccular changes accompanied by mild to moderate phagocytosis and satellitosis, was also observed (Fig. 4.47). Tissue necrosis of variable severity was present in groups given PBZ and MTZ only at the injection sites. These lesions were not found in other treatment groups and the control group.
Figure 4.45: Histomicrograph of liver tissue of a broiler chicken treated with flunixin meglumine showing urate tophi (star) in parenchyma with necrosis (thin arrow) and leukocytic infiltration (thick arrow). 400X.

Figure 4.46: Histomicrograph of myocardium of a broiler chicken treated with flunixin meglumine showing urate crystal deposition (star), tissue necrosis (thin arrow) with leukocytic infiltration (thick arrow). 400X.
Figure 4.47: Histomicrograph of skeletal muscle of a broiler chicken treated with flunixin meglumine showing urate crystal deposition (star) leukocyte infiltration (thick arrow) and myodegeneration (thin arrow). 400X.

Figure 4.48: Histomicrograph of hepatic tissue of a broiler chicken treated with phenylbutazone, showing hepatocyte necrosis (thick arrow). 200X.
Figure 4.49: Histomicrograph of hepatic tissue of a broiler chicken treated with metamizole showing hepatocyte necrosis (thick arrow). 200X.

Figure 4.50: Histomicrograph of hepatic tissue of a broiler chicken treated with ketoprofen showing mild necrosis. 400X.
**Spleen:**

Many urate tophi were scattered in the splenic parenchyma of the affected birds in groups given DF and FM. No histopathological abnormality was observed in the organs of birds of the other treatment groups or the control group.

**Serum Biochemical Analyses**

**Serum Uric Acid**

Mean ± SD values of serum uric acid in all the groups are presented in Table-4.19. The values of serum uric acid in birds of the DF and FM groups were significantly higher than those of control groups at 24, 48 and 72 hours post-exposure, but at 96 hours these values were significantly different from those of the control only for the group given DF. A comparison of uric acid values of all the groups is graphically presented in Fig. 4.51.

### Table-4.19: Serum uric acid concentrations (mg/dL) estimated at different time intervals in broiler chickens treated with various non-steroidal anti-inflammatory drugs (Mean ± SD)

<table>
<thead>
<tr>
<th>Time</th>
<th>C</th>
<th>PBZ</th>
<th>MTZ</th>
<th>KP</th>
<th>MX</th>
<th>PX</th>
<th>DF</th>
<th>FM</th>
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<td>6.25***</td>
</tr>
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<td>5.38***</td>
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<td>5.15±</td>
<td>5.23±</td>
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<td>0.82</td>
<td>0.72</td>
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<td>2.49</td>
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* p<0.05  
*** p<0.001  
C= Control, PBZ= Phenylbutazone, MTZ= Metamizole, KP= Ketoprofen, MX= Meloxicam, PX= Piroxicam, DF= Diclofenac, FM= Flunixin meglumine
Figure 4.51: Serum uric acid concentrations (Mean±SD) in different groups of broiler chickens treated with different non-steroidal anti-inflammatory drugs

Serum Creatinine

In the DF group, serum creatinine values at 24, 48 and 72 hours were significantly different from the control group, while with FM a significant difference was observed only at 48 and 72 hours after the start of dosing period ($p<0.05$).

No significant difference from controls was recorded in the other groups throughout the observation period (Fig 4.52). Mean ± SD values of serum creatinine in the different treatment and the control group are depicted in Table-4.20.
Table 4.20: Serum creatinine concentrations (mg/dL) estimated at different time intervals in broiler chickens treated with various non-steroidal anti-inflammatory drugs

<table>
<thead>
<tr>
<th>Time</th>
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<th>PBZ</th>
<th>MTZ</th>
<th>KP</th>
<th>MX</th>
<th>PX</th>
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<td>0.53±0.14</td>
<td>0.95±0.16**</td>
<td>0.85±0.24*</td>
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<td>0.46±0.06</td>
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* p<0.05
** p<0.01
*** p<0.001

C = Control, PBZ = Phenylbutazone, MTZ = Metamizole, KP = Ketoprofen, MX = Meloxicam, PX = Piroxicam, DF = Diclofenac, FM = Flunixin meglumine

Seum creatinine concentrations

Figure 4.52: Serum creatinine concentrations (Mean±SD) in different groups of broiler chickens treated with various non-steroidal anti-inflammatory drugs
Serum AST

Serum AST activities at 24 hours after the first administration of drugs were significantly higher in the DF and FM groups than in the control group \( (p<0.05) \). At 48 hours, in the PBZ, MTZ, DF, and FN groups, the activities of this enzyme were significantly greater than controls. In the groups given PBZ, MTZ and DF, there was a marked increase in AST activities at 72 and 96 hours of the observation period. The mean ± SD values of serum AST activity are shown in Table-4.21. Changing trends in the activities of AST at different time intervals are shown in Fig. 4.53.
Table 4.21: Serum AST activities (U/L) estimated at different time intervals in broiler chickens treated with various non-steroidal anti-inflammatory drugs

<table>
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<tr>
<th>Time</th>
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<th>MTZ</th>
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<td>11.86</td>
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*p<0.05
**p<0.01
***p<0.001

C= Control, PBZ= Phenylbutazone, MTZ= Metamizole, KP= Ketoprofen, MX= Meloxicam, PX= Piroxicam, DF= Diclofenac, FM= Flunixin meglumine
**Serum ALT**

As compared to controls there was a significant increase in the activities of serum ALT in the samples collected after the initiation of treatment at all time points for the groups given PBZ, MTZ, DF and FM \((p<0.05)\). A significant difference between PX and control groups was observed at 72 hours of the observation period (Fig. 4.54). Mean ± SD values of the enzyme are shown in Table- 4.22.
Table-4.22: Serum ALT activities (U/L) estimated at different time intervals in broiler chickens treated with various non-steroidal anti-inflammatory drugs

<table>
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<tr>
<th>Time</th>
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<th>MTZ</th>
<th>KP</th>
<th>MX</th>
<th>PX</th>
<th>DF</th>
<th>FM</th>
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<td>23.63±</td>
<td>22.94±</td>
<td>24.25±</td>
<td>20.37±</td>
<td>23.45±</td>
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<tr>
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<td>4.72</td>
<td>2.67</td>
<td>2.08</td>
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<td>2.32</td>
<td>4.86</td>
<td>3.52</td>
<td>5.14</td>
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<td>3.73</td>
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<td>4.42</td>
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<td>4.05</td>
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<td>4.87***</td>
<td>3.9</td>
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<td>2.31</td>
<td>12.86***</td>
<td>9.47***</td>
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<td>87.45±</td>
<td>72.56±</td>
<td>25.42±</td>
<td>24.15±</td>
<td>29.63±</td>
<td>47.41±</td>
<td>43.78±</td>
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<tr>
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<td>7.28***</td>
<td>6.34***</td>
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<td>83.77±</td>
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<td>4.73</td>
<td>5.47***</td>
<td>4.57***</td>
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* p<0.05  
** p<0.01  
*** p<0.001

C= Control, PBZ= Phenylbutazone, MTZ= Metamizole, KP= Ketoprofen, MX= Meloxicam, PX= Piroxicam, DF= Diclofenac, FM= Flunixin meglumine

Figure 4.54: Serum ALT activities (Mean±SD) in different groups of broiler chickens treated with various non-steroidal anti-inflammatory drugs
**Serum ALP**

Before the start of experiment, serum ALP values were similar in the birds of the control and treatment groups. Mean ± SD values of the enzyme are shown in Table-4.23. The enzyme activities were significantly higher in the groups given PBZ and MTZ at all post-exposure sampling points \((p<0.05)\). A significant rise in the enzyme activity was observed at 24 and 48 hours in groups given DF and FN. Fig. 4.55 shows the comparison of serum ALP activities among the control and the different treatment groups.
Table 4.23: Serum ALP activities (U/L) estimated at different time intervals in broiler chickens treated with various non-steroidal anti-inflammatory drugs

(Mean ± SD)

<table>
<thead>
<tr>
<th>Time</th>
<th>C</th>
<th>PBZ</th>
<th>MTZ</th>
<th>KP</th>
<th>MX</th>
<th>PX</th>
<th>DF</th>
<th>FM</th>
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<td>534.82±34.36</td>
<td>530.28±25.47</td>
<td>534.53±37.35</td>
<td>524.25±40.31</td>
</tr>
<tr>
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<td>632.25±63.56***</td>
<td>619.15±34.5**</td>
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<td>535.38±27.45</td>
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<td>544.09±19.82</td>
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<td>646.33±58.44***</td>
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<td>827.35±104.36***</td>
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<tr>
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<td>536.47±34.1</td>
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</tbody>
</table>

** p<0.01  
*** p<0.001  
C= Control, PBZ= Phenylbutazone, MTZ= Metamizole, KP= Ketoprofen, MX= Meloxicam, PX= Piroxicam, DF= Diclofenac, FM= Flunixin meglumine

Serum alkaline phosphatase activities

Figure 4.55: Serum ALP activities (Mean±SD) in different groups of broiler chickens treated with various non-steroidal anti-inflammatory drugs
EXPERIMENT NO. 4

Efficacy Trials of Non-steroidal Anti-inflammatory Drugs in Experimentally-induced Pyrexia in Buffalo Calves

In order to evaluate the efficacy of the safer NSAIDs that may be used as alternatives to diclofenac, six groups of buffalo calves, each with five animals, were arranged and labeled as groups 1 to 6.

Group 1 was neither exposed to LPS nor treated with any drug. Animals in group 2 were given LPS to induce pyrexia but remained untreated. The groups from no. 3 to 6 were first injected with LPS and later treated with different NSAIDs, i.e. diclofenac, ketoprofen, meloxicam and piroxicam, respectively.

The following clinical and laboratory parameters were studied in all six groups;

- Rectal Temperature
- Respiration Rate
- Pulse Rate
- Total Red Blood Cell Count
- Total Leukocyte Count
- Platelet Count
- Hemoglobin (Hb)
- Hematocrit (PCV)
- Total Protein (TP)
- Creatine Kinase (CK)
- Aspartate Amino-transaminase (AST)
- Creatinine (CREA)
Clinical Parameters

The animals in groups 2 to 6 showed moderate depression after the injection of LPS. Body temperatures, respiration rates and pulse rates increased significantly in LPS-dosed animals compared with the animals in the environment control (EC) group. No clinical abnormality was observed in the animals of the EC group throughout the experiment.

Rectal Temperature

Rectal temperatures were recorded before the start of the experiment and at one-hour intervals afterwards for 15 hours. Temperature values of all the animals of the treatment and control groups were in the normal range before the start of experiment. In group 1 (EC), no significant difference was observed at all the time points. A highly significant rise in body temperature in group 2 (LPS control) was observed at all the points except at 0 and 15 hours of the observation period when compared with group 1. Animals in all treatment groups were treated with respective drugs 3 hours after the exposure to LPS. The fall in body temperature in all the treatment groups was compared to that in group 2. A significant decrease in the temperature was recorded in groups 3 and 4, two hours post-treatment, while such change was noticed after three hours in groups 5 and 6. No significant difference was recorded between the responses between groups 3 and 4 or between groups 5 and 6 (Fig 4.56). Mean ± SD values of rectal temperatures are presented in Table-4.24.
Table 4.24: Rectal temperature (°C) taken at different time intervals in various groups of buffalo calves treated with different non-steroidal anti-inflammatory drugs following *Escherichia coli* lipopolysaccharide endotoxin induced pyrexia (Mean ± SD)

<table>
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<th>Time (h)</th>
<th>Env. C</th>
<th>LPS control</th>
<th>DF</th>
<th>KP</th>
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</tr>
<tr>
<td>2</td>
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<td>39.64±0.18</td>
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<td>38.56±0.11</td>
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<td>38.36±0.05</td>
<td>38.34±0.05</td>
<td>38.34±0.11</td>
</tr>
</tbody>
</table>

**Env. C= Environmental Control, DF= Diclofenac, KP= Ketoprofen, MX= Meloxicam, PX= Piroxicam**

**Exp 4: Rectal Temperatures**

![Graph showing responses of rectal temperatures (Mean ± SD) to various treatments with non-steroidal anti-inflammatory drugs in lipopolysaccharide endotoxin-induced pyrexia in buffalo calves. NS= normal saline, LPS= lipopolysaccharide](image-url)

Figure 4.56: Graph showing responses of rectal temperatures (Mean ± SD) to various treatments with non-steroidal anti-inflammatory drugs in lipopolysaccharide endotoxin-induced pyrexia in buffalo calves. NS= normal saline, LPS= lipopolysaccharide
Respiration Rate

Respiration rates of the experimental animals were recorded in the control and treatment groups initially just before the start of experiment then at 3, 6, 9, 12 and 15 hours of the observation period. The initial measurements at 0 hour were not significantly different among all the groups. A significant increase in respiration rates in group 2 was observed from 3 hours onward in comparison with group 1. For the first 3 hours after of LPS dosing there was no significant difference between group 2 and all the treatment groups. Thereafter, in all the treatment groups, there were significantly lower respiration rates as compared to the LPS control group (Group 2). Mean ± SD values of respiration rate in all the experiment groups are presented in Table-4.25. Changes in the respiration rates in various groups at different time points are shown in Fig. 4.57.
Table 4.25: Effect of different treatment regimens on respiration rates (per minute) in *Escherichia coli* lipopolysaccharide endotoxin-treated buffalo calves

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Env. C</th>
<th>LPS control</th>
<th>DF</th>
<th>KP</th>
<th>MX</th>
<th>PX</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>33.6±1.7</td>
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<td>32.9±2.8</td>
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<td>47.4±1.6</td>
<td>45.1±1.4</td>
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</tr>
<tr>
<td>6</td>
<td>33.2±1.4</td>
<td>54.7±2.1</td>
<td>35.2±2.8***</td>
<td>35.3±1.6***</td>
<td>49.2±1.4***</td>
<td>48.2±1.9***</td>
</tr>
<tr>
<td>9</td>
<td>33.8±0.8</td>
<td>51.6±1.5</td>
<td>34.4±1.3***</td>
<td>34.7±1.9***</td>
<td>46.7±1.7***</td>
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<td>15</td>
<td>33.3±1.7</td>
<td>36.9±1.9</td>
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<td>33.6±1.2*</td>
<td>33.7±1.1*</td>
<td>33.6±1*</td>
</tr>
</tbody>
</table>

*p<0.05

***p<0.001

Env. C= Environmental Control, DF= Diclofenac, KP= Ketoprofen, MX= Meloxicam, PX= Piroxicam

Figure 4.57: Graphic representation of the effects of different non-steroidal anti-inflammatory drugs treatments on respiration rates (Mean ± SD) in different buffalo calf groups. NS= normal saline, LPS= lipopolysaccharide
Pulse Rate

The mean±SD values of pulse rates in the animals of different groups before and after the start of LPS administration are shown in Table-4.26. There was no significant difference observed between groups 1 and 2 at all the time points. The pulse rates of groups 3 and 4 were not significantly different from one another, but they were significantly lower than those of group 2 at 3, 6, 9 and 12 hours. Significantly lower pulse rates were observed in groups 5 and 6 as compared to the untreated control group at 9 and 12, hours but no significant difference was present between groups 5 and 6. Changes in the pulse rates in various groups are presented in Fig 4.58.
Table 4.26: Effect of different treatment regimens on pulse rates (per minute) in *Escherichia coli* lipopolysaccharide endotoxin-treated buffalo calves

(Mean±SD)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Env. C</th>
<th>LPS control</th>
<th>DF</th>
<th>KP</th>
<th>MX</th>
<th>PX</th>
</tr>
</thead>
<tbody>
<tr>
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<td>76.4±3.6</td>
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<td>73.7±4</td>
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</tr>
<tr>
<td>3</td>
<td>75.1±4.7</td>
<td>86.5±5.8</td>
<td>88.7±4.6***</td>
<td>88.4±4.5***</td>
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<td>87.3±3.7</td>
</tr>
<tr>
<td>6</td>
<td>72.9±4.3</td>
<td>92.4±6.4</td>
<td>81.2±5.6***</td>
<td>79.5±4.8***</td>
<td>90.4±4.3</td>
<td>91.7±4.4</td>
</tr>
<tr>
<td>9</td>
<td>76.4±5.5</td>
<td>94.7±5.3</td>
<td>78.4±3.9***</td>
<td>74.6±3.7***</td>
<td>80.2±3.8***</td>
<td>81.4±3.8***</td>
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<td>77.9±4.5</td>
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<td>73.8±4.1***</td>
<td>76.4±4.5**</td>
<td>77.5±4.6</td>
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<tr>
<td>15</td>
<td>75.3±3.8</td>
<td>79.4±3.6</td>
<td>73.4±4.2</td>
<td>72.6±3.3</td>
<td>74.8±4</td>
<td>76.3±3.6</td>
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</table>

*p<0.05
** p<0.01
*** p<0.001

Env. C= Environmental Control, DF= Diclofenac, KP= Ketoprofen, MX= Meloxicam, PX= Piroxicam

Figure 4.58: Graphic comparisons of the effects of different non-steroidal anti-inflammatory treatments on pulse rates in different buffalo calf groups. NS= normal saline, LPS= lipopolysaccharide
Hematology

The total erythrocyte counts, platelet counts and hemoglobin concentration (Hb) remained in normal ranges in samples collected from the different groups at all time intervals, and no significant difference was found in these values throughout the observation period ($p<0.05$).

Total Leukocyte Count

The total leukocyte counts (TLCs) from the different groups before initiation of the experiment did not differ significantly from each other. A highly significant reduction in TLCs was recorded in group 2 in comparison with group 1 from 3 to 15 hours. TLC values in all the treatment groups were similar to that in group 2 throughout the observation period. Mean ± SD values of TLC are given in Table-4.27. Total leukocyte counts as a function of treatment and time are shown in Fig. 4.59.
Table 4.27: Effect of different treatment regimens on total leukocyte counts (10^3/ml) in *Escherichia coli* lipopolysaccharide endotoxin-treated buffalo calves

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Env. C</th>
<th>LPS control</th>
<th>DF</th>
<th>KP</th>
<th>MX</th>
<th>PX</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>12.878±1.809</td>
<td>1.462±0.377</td>
<td>1.35±0.347</td>
<td>1.52±0.240</td>
<td>1.17±0.191</td>
<td>1.72±0.232</td>
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<td>12.998±1.468</td>
<td>2.45±0.586</td>
<td>2.882±0.506</td>
<td>3.5±0.467</td>
<td>2.714±0.372</td>
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<td>13.182±1.676</td>
<td>4.238±0.749</td>
<td>5.114±0.246</td>
<td>6.282±0.968*</td>
<td>4.814±0.406</td>
<td>4.724±0.338</td>
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<tr>
<td>12</td>
<td>13.074±1.526</td>
<td>9.002±1.087</td>
<td>9.246±0.693</td>
<td>9.782±0.422</td>
<td>9.148±0.615</td>
<td>8.582±0.282</td>
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</table>

*p<0.05

Env. C= Environmental Control, DF= Diclofenac, KP= Ketoprofen, MX= Meloxicam, PX= Piroxicam

Figure 4.59: Graphic comparison of the effect of different non-steroidal anti-inflammatory drugs treatments on total leukocyte counts (Mean ± SD) in different buffalo calf groups at different time intervals. NS= normal saline, LPS= lipopolysaccharide
Hematocrit

No significant difference was observed in hematocrit values for the animals of all the groups at t=0. These values increased significantly in the LPS control group (Group 2) throughout the observation period. A significant difference was observed for all the treatment groups when compared to the LPS control group 6 hours and onward ($p<0.05$). The mean ± SD for hematocrit values of all the groups are depicted in Table-4.28. The trends in changing hematocrit values are illustrated in Fig. 4.60.
Table 4.28: Effects of different treatment regimens on hematocrit values (%) in *Escherichia coli* lipopolysaccharide endotoxin-treated buffalo calves

<table>
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<tr>
<th>Time (h)</th>
<th>Env. C (Mean ± SD)</th>
<th>LPS control (Mean ± SD)</th>
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<th>KP (Mean ± SD)</th>
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<th>PX (Mean ± SD)</th>
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<td>29.95±1.3</td>
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<tr>
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<td>28.55±1.62</td>
<td>35.52±1.82</td>
<td>32.37±1.05***</td>
<td>31.57±1.04***</td>
<td>33.78±0.85***</td>
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<tr>
<td>9</td>
<td>29.03±0.74</td>
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<td>29.82±0.83***</td>
<td>32.52±0.96***</td>
<td>32.69±1.05***</td>
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<tr>
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<td>27.7±1.22***</td>
<td>30.71±1.02***</td>
<td>31.1±0.84***</td>
</tr>
<tr>
<td>15</td>
<td>27.58±1.17</td>
<td>37.29±1.95</td>
<td>28.01±1.14***</td>
<td>26.93±1.14***</td>
<td>29.38±0.87***</td>
<td>29.25±1.13***</td>
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</tbody>
</table>

p<0.001

Env. C= Environmental Control, DF= Diclofenac, KP= Ketoprofen, MX= Meloxicam, PX= Piroxicam

Figure 4.60: Comparison of hematocrit percentage (Mean ± SD) in buffalo calves of control, LPS and LPS plus different non-steroidal anti-inflammatory drugs treatment groups.

NS= normal saline, LPS= lipopolysaccharide
Total Serum Protein

Total serum protein values did not differ significantly among all the groups prior the exposure to LPS. A significant decrease in the values of total protein was recorded in the animals of LPS control group compared to the environmental control group from 3 to 15 hours of experiment. In groups 3 and 4, a significant improvement in total protein concentration was noted as compared to group 2 from 6 hours until the end of observation period, while, in groups 5 and 6, a similar pattern in the recovery of total protein concentration was observed from 9 hours to 15 hours. It was noted that the total serum protein values of group 3 did not differ significantly at any time from group 4. Also group 5 did not differ significantly from group 6 at any time \((p<0.05)\). Mean ± SD values of total serum protein are shown in Table-4.29. These values are graphically presented in Fig 4.61.
**Table 4.29:** Effects of different treatment regimens on total serum protein (g/L) in *Escherichia coli* lipopolysaccharide endotoxin-treated buffalo calves

(Mean±SD)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Env. C</th>
<th>LPS control</th>
<th>DF</th>
<th>KP</th>
<th>MX</th>
<th>PX</th>
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<td>6.75±0.07</td>
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<td>6.45±0.09</td>
</tr>
<tr>
<td>6</td>
<td>7.6±0.33</td>
<td>5.36±0.08</td>
<td>6.46±0.12 ***</td>
<td>6.33±0.1 ***</td>
<td>5.47±0.17</td>
<td>5.53±0.13</td>
</tr>
<tr>
<td>9</td>
<td>7.46±0.14</td>
<td>4.58±0.2</td>
<td>6.94±0.09 ***</td>
<td>7.02±0.15 ***</td>
<td>6.35±0.08 ***</td>
<td>6.71±0.1 ***</td>
</tr>
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<td>3.82±0.18</td>
<td>7.47±0.18 ***</td>
<td>7.55±0.08 ***</td>
<td>6.96±0.12 ***</td>
<td>7.02±0.16 ***</td>
</tr>
<tr>
<td>15</td>
<td>7.45±0.16</td>
<td>3.85±0.23</td>
<td>7.46±0.11 ***</td>
<td>7.52±0.2 ***</td>
<td>7.54±0.14 ***</td>
<td>7.58±0.11 ***</td>
</tr>
</tbody>
</table>

*** p<0.001

Env. C= Environmental Control, DF= Diclofenac, KP= Ketoprofen, MX= Meloxicam, PX= Piroxicam

**Figure 4.61:** Total serum protein concentrations (Mean ± SD) over time in various buffalo calf treatment groups. NS= normal saline, LPS= lipopolysaccharide
Serum Creatinine

Serum creatinine concentrations in blood samples of all the groups collected before the start of experiment were in the normal range. The comparison of creatinine values between groups 1 and 2 revealed that a significant decrease occurred in LPS control group (group 2) from 3 hours post-exposure until the end of observation period. In groups 3 and 4, there was a significant decline in creatinine concentration from that in group 2 from 6 hours to 15 hours. By contrast, compared to group 2 (LPS-dosed controls), groups 5 and 6 had significantly smaller reductions in creatinine concentrations from 9 hours until the end of experiment ($p<0.05$). Mean ± SD serum creatinine values are exhibited in Table-4.30 and Fig. 4.62.
Table 4.30: Responses of serum creatinine concentration (mg/dL) to different treatment regimens in *Escherichia coli* lipopolysaccharide endotoxin-treated buffalo calves

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Env. C</th>
<th>LPS control</th>
<th>DF</th>
<th>KP</th>
<th>MX</th>
<th>PX</th>
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<tbody>
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<td>1.76±0.14</td>
<td>1.56±0.08</td>
<td>1.59±0.09</td>
<td>1.56±0.08</td>
<td>1.53±0.1</td>
<td>1.54±0.07</td>
</tr>
<tr>
<td>6</td>
<td>1.71±0.08</td>
<td>1.47±0.1</td>
<td>1.66±0.1</td>
<td>1.64±0.07*</td>
<td>1.57±0.08**</td>
<td>1.57±0.1</td>
</tr>
<tr>
<td>9</td>
<td>1.78±0.06</td>
<td>1.35±0.09</td>
<td>1.78±0.08***</td>
<td>1.73±0.09***</td>
<td>1.6±0.07***</td>
<td>1.61±0.08***</td>
</tr>
<tr>
<td>12</td>
<td>1.74±0.11</td>
<td>1.28±0.11</td>
<td>1.81±0.07***</td>
<td>1.76±0.07***</td>
<td>1.65±0.12***</td>
<td>1.67±0.11***</td>
</tr>
<tr>
<td>15</td>
<td>1.73±0.12</td>
<td>1.36±0.07</td>
<td>1.83±0.09***</td>
<td>1.80±0.11***</td>
<td>1.69±0.09***</td>
<td>1.75±0.07***</td>
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</table>

*P<0.05  *** p<0.001

Env. C= Environmental Control, DF= Diclofenac, KP= Ketoprofen, MX= Meloxicam, PX= Piroxicam

---

**Figure 4.62:** Mean serum creatinine concentrations (Mean ± SD) of the treatment groups of buffalo calves at different time points. NS= normal saline, LPS= lipopolysaccharide
Serum AST

Mean ± SD values of serum AST are presented in Table-4.31. Serum AST activity in the samples collected from all the control and treatment groups at 0 hour did not differ significantly. After the exposure to LPS, a significant increase in the activity of AST was observed in the LPS control group (Group 2) when compared with that in the environmental control group (Group 1) after the start of experiment. However, the mean values of serum AST activities were significantly lower in other groups when compared to those of group 2 from six hours to 15 hours after dosing (p<0.05). Fluctuations in AST activity at various time points are depicted in Fig. 4.63.
Table 4.31: Responses of serum aspartate aminotransferase (U/L) to different treatment regimens in *Escherichia coli* lipopolysaccharide endotoxin-treated buffalo calves

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Env. C</th>
<th>LPS control</th>
<th>DF</th>
<th>KP</th>
<th>MX</th>
<th>PX</th>
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<td>292.1±10.34***</td>
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<td>192.62±11.33***</td>
<td>258.68±11.03***</td>
<td>255.72±11.15***</td>
</tr>
</tbody>
</table>

*** p<0.001

Env. C= Environmental Control, DF= Diclofenac, KP= Ketoprofen, MX= Meloxicam, PX= Piroxicam

![Serum Aspartate aminotransferase Activities](image)

Figure 4.63: Graphic comparison of serum aspartate aminotransferase activities (Mean ± SD) in different experimental groups of buffalo calves treated with *Escherichia coli* lipopolysaccharide and non-steroidal anti-inflammatory drugs. NS= normal saline, LPS= lipopolysaccharide
Creatine Kinase

Creatine kinase activity in the serum samples collected before the start of the experiment from different groups were in the normal range and did not differ significantly from each other. At all time points thereafter, the values of group 2 were significantly greater than group 1 \((p<0.05)\). Mean ± SD values of serum creatine kinase are shown in Table-4.32. Activities of this particular enzyme were significantly lower in all the treatment groups (Groups 3-6) compared to group 2 from 6 hours onwards. The graphic representation of the pattern of the changes in enzyme activities is presented in Fig 4.64.
Table 4.32: Serum creatine kinase (U/L) activity in environmental controls, and various treatment groups of buffalo calves given *Escherichia coli* lipopolysaccharide as a function of time.

(\textit{Mean ± SD})

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Env. C</th>
<th>LPS control</th>
<th>DF</th>
<th>KP</th>
<th>MX</th>
<th>PX</th>
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</tr>
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<td>15</td>
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<td>89.36±7.48***</td>
<td>122.6±7.82***</td>
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</tr>
</tbody>
</table>

**p<0.001

Env. C= Environmental Control, DF= Diclofenac, KP= Ketoprofen, MX= Meloxicam, PX= Piroxicam

**Figure 4.64:** Comparison of creatine kinase (CK) activities (Mean± SD) among different groups of buffalo calves treated with different non-steroidal anti-inflammatory drugs subsequent to *Escherichia coli* lipopolysaccharide-induced pyrexia. NS= normal saline, LPS= lipopolysaccharide
Chapter 5

DISCUSSION

Non-steroidal anti-inflammatory drugs (NSAIDs) are a group of chemically heterogeneous compounds used for more or less similar therapeutic indications. All NSAIDs, including selective COX-2 inhibitors, are antipyretic, analgesic, and anti-inflammatory, with the exception of acetaminophen, which is antipyretic and analgesic but is largely devoid of antiinflammatory activity. Aspirin is the prototype NSAID. It is the first drug of this group and still widely used, despite development of newer and in some cases more potent compounds (FitzGerald et al., 2007). NSAIDs find their main clinical application as antiinflammatory agents in the treatment of musculoskeletal disorders, such as rheumatoid arthritis and osteoarthritis (Chatterjee et al., 1996). In general, NSAIDs provide only symptomatic relief from pain and inflammation associated with the disease, but do not arrest the progression of pathological injury to the tissue, and are not considered to be "disease-modifying" anti-rheumatic drugs.

NSAIDs are prescribed for pain of low to moderate intensity, such as dental pain. These drugs are also valuable in orthopedic and soft tissue surgical procedures, especially where extensive inflammation and soft tissue trauma are present. Other clinical conditions in humans, where NSAIDs are prescribed include postoperative pain, dysmenorrhea, patent ductus arteriosus, systemic mastocytosis and cancer chemoprevention (Anne et al., 2005). In veterinary practice, these drugs are used to manage pain, including postoperative pain, and pain associated with both arthritis and colic. NSAIDs are also used in animals, along with antimicrobial drugs, as an adjunct therapy to alleviate pain and inflammation (Lees, 2009).
Major adverse effects of NSAIDs observed in humans and other animals include gastrointestinal disorders, most notably mucosal damage, bleeding, ulceration, and perforation. Other adverse effects reported include nephrotoxicity, hepatotoxicity, blood clotting disorders and allergic reactions.

A unique toxic effect of diclofenac in vultures was reported when they ingested residues of the drug in carcasses of dead animals that had been treated shortly before death (Oaks et al., 2004). It was observed that a sharp decline of at least three vulture species occurred in Southeast Asian countries including India, Pakistan, Nepal and Bangladesh (Prakash, 1999; Prakash et al., 2001; Oaks et al., 2004; Gilbert et al., 2002). A rapid and massive decrease in vulture population was confirmed by the IUCN, which declared three vulture species as ‘critically endangered’ (Green et al., 2007). The decrease in the numbers of vultures has harmed the region’s ecosystems and the environments. With vast reductions in vultures, other scavengers including stray dogs have increased. The increase in stray dogs, in turn, has increased risks of communicable diseases, including zoonoses. For example, the risk of rabies has greatly increased (Cunningham et al., 2001; Prakash et al., 2003). Experimental studies on vultures proved that Gyps bengalensis is extremely sensitive to diclofenac toxicity (Oaks et al., 2004).

In order to find alternative NSAIDs to diclofenac, that are safer for scavenging birds and efficacious in target animals, five experiments were designed and completed in two phases. In the first phase, a chicken experimental model was developed to study the toxicity of various NSAIDs. Different drugs of this class were screened to establish toxicity profiles in comparison to diclofenac. In the second phase, studies of NSAIDs that were safer in chickens
were examined for comparative therapeutic efficacy using target species under experimental and clinical situations in buffaloes and horses, respectively.

Keeping in view the non-availability of the endangered vultures for experimental purposes, different bird species, including black kites, crows, pigeons, and broiler chickens were considered as potential experimental models. Broiler chickens were chosen due to easy availability, trouble-free handling and well-studied physiological parameters. Different doses of diclofenac sodium were administered to the birds of various groups intramuscularly or orally for four consecutive days. Twenty-four hours post-exposure, the birds in the higher dose groups, i.e. 5 mg/kg and 10 mg/kg body weight, started exhibiting clinical signs of mild to moderate depression followed by lethargy, anorexia, and reluctance to move. According to Lumeij (1994), in cases where renal urate deposition occurs prior to visceral gout, anorexia and lethargy may be noted for hours or days. Depression became severe with the passage of time and the affected birds stopped moving. In the advanced stages of toxicosis, the birds completely stopped eating and drinking, perched aside from the other birds, failed to hold their necks in normal posture, sat down with closed eyes, and ultimately died in the same position. Similarly, severe depression and lethargy characterized by a syndrome involving drooping heads had been observed in vultures, which were affected by diclofenac toxicosis either in the field or experimentally. These vultures were apparently unable to hold their necks in the normal position (Prakash et al., 2003; Swan et al., 2005). Similar clinical findings from diclofenac toxicosis have also been reported in studies of experimentally dosed domestic fowl (Naidoo et al., 2007). According to Speer (1997), birds experienced signs of lethargy, weakness and anorexia as a consequence of renal disease. Although the pH of the blood was not measured in this study, the development of depression in affected birds can
likely be explained, at least in part, from metabolic acidosis, which may result from observed increases in serum uric acid, and this may be aggravated by reduced ability of proximal convoluted tubules to conserve bicarbonate (Seifter, 2004). Acidosis, similarly, leads to CNS depression in cattle and people (Blood and Radostits, 1989; Seifter, 2004). In this study, sudden death in the affected chickens was recorded. The acidosis was consistent with the observed increase in blood uric acid, and acidosis may result in hyperkalemia, which in turn might lead to cardiac arrest and sudden death (Lierz, 2003).

The mortality ratios in chickens in the current study that were given diclofenac were significantly higher after intramuscular dosing compared to those treated via the oral route. This observation suggests that the bioavailability of diclofenac when administered orally may be lower than that of the intramuscular route. A 50% bioavailability of diclofenac was observed in fowl after oral dosing (Naidoo et al., 2007). In the present study, mortality rates of 40% and 30% were recorded at 5 mg/kg following intramuscular and oral administration, respectively. In two separate studies, 33% and 40% mortality rates were reported with diclofenac at the same dose in layer and broiler chicken, respectively (Reddy et al., 2006; Naidoo et al, 2007). In present study, no mortality occurred in broiler chickens given diclofenac at 1.25 mg/kg, whilst 100% mortality was reported in Gyps bengalensis vultures at doses as low as 0.8 mg/kg (Oaks et al., 2004). Vultures clearly seem to be more sensitive to diclofenac toxicity than broiler chickens. This difference in susceptibility may be due to differences between the species in fate or receptor activity.

Postmortem examinations conducted on the chickens that died due to diclofenac toxicity revealed that visceral gout was the most consistent postmortem lesion. It was distinctly evident in almost all the dead chickens of the various groups. Visceral gout was also
observed as the main postmortem lesion in dead vultures collected from the wild and in those treated with diclofenac either orally or through meat of diclofenac-treated animals (Gilbert et al., 2002; Cunningham et al., 2003; Oaks et al., 2004; Swan et al., 2005). A strong correlation was established between the presence of visceral gout and detection of diclofenac residues in body tissues of the affected vultures (Oaks et al., 2004; Shultz et al., 2004). Urate crystals were extensively precipitated on the surface and within the parenchyma of the organs of vultures with renal failure (Meteyer et al., 2005). Visceral gout was also evident in a study of domestic fowl treated with diclofenac (Naidoo et al., 2007). It is suggested that damage to the kidney tissue from diclofenac may interfere with the normal excretion of uric acid from blood resulting in hyperuricemia. The excessive uric acid ultimately starts depositing inside and on the visceral organs resulting in the condition known as visceral gout (Lumeij, 1994). It has been reported that high dietary protein and calcium intake in birds may also cause hyperuricemia (Lumeij, 1994; Guo et al., 2005). Other studies showed that sodium bicarbonate can cause gout in broiler and layer chickens (Mubarak and Sharkawy, 1999; Ejaz et al., 2005).

Excessive protein, calcium and sodium bicarbonate would not explain the observed development of visceral gout in the present study because the same diet was fed to all the treatment as well as control groups, and visceral gout was not observed in the birds of the control groups.

Crespo and Shivaprasad (2003) stated that visceral gout is caused by renal failure. The kidneys may fail as a result of metabolic, infectious, or toxicologic diseases. In current study, during necropsy of the diclofenac-dosed broiler chickens it was observed that kidneys were swollen and pale and liver was friable. The nonsteroidal anti-inflammatory agent flunixin has
been reported to cause nephrotoxicity in cranes and flamingos as well as gout in northern bobwhite quail \textit{[Colinus virginianus]} (Klein \textit{et al.}, 1994).

Histopathology was performed on kidney, liver, spleen, heart, and skeletal muscles collected from the chickens of all treatment and control groups in the current study. Histomicrographs of kidney tissues of the affected birds revealed extensive necrosis of tubules with deposition of urate crystals in the form of urate tophi damaging the normal architecture of the kidney tissues. Mild to moderate inflammatory reaction was evident in the kidney tissues of affected birds. Most of the convoluted tubules contained cellular casts and debris. Glomeruli appeared to be spared. In liver, focal necrosis of hepatocytes was accompanied by infiltrations of inflammatory cells. Urate tophi deposition was also evident. Similarly, the deposition of urate crystals was associated with inflammatory reactions and necrosis in heart, spleen, and skeletal muscle tissues.

The results of this study are fully supported by the findings of a previous study, in which it was reported that a low frequency of glomerular congestion, deposition of immunocomplexes, and tubular necrosis were observed in budgerigars treated with NSAIDs for three days (Pereira and Werther, 2007). As shown in this study, Meteyer \textit{et al.}, (2005) reported that acute proximal tubular necrosis was observed in the \textit{Gyps bengalensis} vultures that died due to the toxicity of diclofenac. They reported that such lesions became extensive, and that large urate aggregates obscured the normal renal structure. Less extensive damage was noted in distal tubule and glomeruli. Uric acid crystals were also seen commonly in liver, spleen, lung, and heart. These finding are congruent with those of present study. The results of this study are consistent with the observations that renal tubular necrosis in humans is associated with the use of NSAIDs and results in renal failure (McCrory \textit{et al.}, 2002).
Aydin et al., (2003) reported that diclofenac produced histopathological changes in the liver and kidney of rats that were similar to those observed in present study, however, they recorded cloudy swelling, degeneration and fibrosis which were absent in the tissues sections examined during present work. This dissimilarity may be due to the prolonged exposure of the rats to diclofenac, as they survived throughout the observation period. The findings of present study are also partially in line with those in broiler chickens as reported by Reddy et al. (2006). Fibrosis in kidney and hyperplasia in liver was recorded in that study, while these lesions were not observed in this study. The reason for the disagreement may be the longer duration of diclofenac exposure in the Reddy et al study.

The findings of this study are well supported by the results of a study of domestic fowl treated with diclofenac that was presented by Naidoo et al. (2007). These workers reported cell necrosis and tubular loss with massive urate crystal precipitation in kidneys as well as hepatocyte necrosis with small urate tophi. The absence of fibrosis and inflammatory cellular infiltration in the study of Naidoo et al. (2007) and in the current study may be due to shorter times of exposure to toxic doses of diclofenac.

A significant rise in serum uric acid concentrations were observed with higher doses of diclofenac by 24 hours post-exposure whether dosing was via the intramuscular or oral routes. These findings are congruent with those reported from studies of Gyps vultures (Swan et al., 2006). Similar observations were noticed by Naidoo et al. (2007) in domestic fowl after the treatment with diclofenac. By contrast no changes in uric acid concentrations were observed in budgerigars (Pereira and Werther, 2007) and northern bobwhite quails (Klein et al., 1994) treated with flunixin meglumine. This difference in observations may be due to different species and the variable sensitivity to the drug used. Differing explanations for
increases in uric acid concentration in response to kidney damage have been made. Normal levels of uric acid have been observed in renal damage in certain patients (Hochleithner, 1994) and as a consequence of the large volume of uric acid, that they excreted from the tubules independently of the glomerular filtration rate, the concentration of uric acid in the blood did not easily change (Styles and Phalen, 1998) by contrast Fudge (1997) noted that an increase in blood uric acid concentration is indicative of renal disease and, if moderate to high increases are seen, there may be a significant tubular injury.

The findings of the current study with chickens are fully supported by the results of a recent study of oriental white-backed vultures [Gyps bengalensis] in which hyperuricaemia was observed at 24 hours after treatment with both low and high dosages of diclofenac (Oaks et al., 2004).

Serum creatinine concentrations provide another indicator of renal function. In this study, the higher doses of diclofenac administered to broiler chickens via both IM and oral routes were consistently followed by significant increases in serum creatinine concentrations. These findings are completely in agreement with the observations made by Reddy et al. (2006) in broiler chickens after treatment with diclofenac. The findings of the present study are also in line with those reported by Mathews, et al. (1990), who observed significant elevations in serum creatinine concentrations in dogs given flunixin meglumine along with methoxyflurane.

In the current study of chickens it was observed that serum AST, ALT and ALP activities were significantly elevated, and these increases in enzyme activity were dose-dependent. The findings of present study are in harmony with the observations made in chickens treated with diclofenac (Reddy et al., 2006). The results of this study are also in concordance with
those reported by Swan et al. (2006) who observed a significant rise in ALT activity in *Gyps benegalensis* post-exposure to diclofenac. They noticed that the values of serum ALP and AST were significantly elevated in the diclofenac-treated group. A significant rise in ALT was also observed in dogs treated with flunixin meglumine (Mathews et al., 1996), hence these observations are in line with the findings of the present study.

In the second experiment of this thesis, three avian species, i.e. chickens, pigeons, and quails, were compared in regard to their susceptibility to diclofenac toxicity. The study parameters remained the same as in the previous experiment. The results indicated great variation in the severity of toxic effects observed in the different bird species. The clinical signs observed in chickens were identical to those recorded in the first experiment and were similar to those noted in pigeons exposed to toxic doses of diclofenac.

The quail showed no clinical signs of diclofenac toxicosis throughout the observation period. These findings are supported by the study conducted by Lucas (2003) who observed that no renal impairment occurred in Japanese quail treated with flunixin meglumine and meloxicam. Different species of animals vary in the sensitivity to the toxic effects of various drugs. Cats are highly susceptible to the toxicity of acetaminophen (Campbell & Chapman, 2000a) and permethrin (Martin and Campbell, 2000). Similarly some studies indicate that dogs are unusually sensitive to the toxic effects of NSAIDs (Campbell & Chapman, 2000a). Findings of these studies are in line with those observed in present study.

In this study, chickens and pigeons with diclofenac toxicosis showed lethargy, anorexia and weakness. Pereira and Werther (2007), observed no typical signs of NSAID toxicity in budgerigars, and similar findings were recorded in the quails of the present study. These
findings are congruent with those described by Naidoo et al. (2007) in chickens dosed with diclofenac.

In this study, 70% and 40% mortality rates were recorded in the chicken and pigeon groups, respectively. These observations were in line with the findings of many researchers who reported variable mortality rates in vultures (Gyps bengalensis, Gyps africanus) (Oaks et al., 2004; Swan et al., 2006a), broiler chickens (Reddy et al., 2006) and domestic fowl (Gallus domesticus) (Naidoo et al., 2007) when exposed to diclofenac. In this study, great variation was noted in the effects of diclofenac on the chosen avian species. The quail were less sensitive to diclofenac toxicity than pigeons and chickens. No mortality occurred in quail treated with diclofenac. These observations are congruent to the findings of the study in which no mortality was recorded in budgerigars treated with 5·5 mg/kg flunixin meglumine, 2·5 mg/kg ketoprofen, or 0·1 mg/kg meloxicam (Pereira and Werther, 2007).

Postmortem examinations conducted on the dead chickens and pigeons revealed the presence of visceral gout and swelling of kidneys as major necropsy lesions. However, no visceral gout or kidney lesions were found in any quail when examined at the time of necropsy. This indicates that quails are more resistant to the toxicity of diclofenac than chickens and pigeons. Visceral gout after exposure to diclofenac was previously observed in vultures (Gilbert et al., 2002; Cunningham et al., 2003; Oaks et al., 2004; Shultz et al., 2004; Swan et al., 2005) and in domestic fowl (Naidoo et al., 2007).

The histopathologic changes in liver, kidney, heart tissue and skeletal muscles in the chickens and pigeons of the current study were similar to those described in previous experiments. These changes were supported by the findings of many researchers in different species, i.e. mice (Aydin et al., 2003; Yasmeen et al., 2007), Gyps vultures (Meteyer et al.,
2005; Mishra et al., 2002), Fowls (Naidoo et al., 2007), budgerigars (Pereira and Werther, 2007) and broiler chickens (Reddy et al., 2006).

Serum uric acid, creatinine, AST, ALT and ALP values for chickens and pigeons were significantly elevated in comparison with those for quails. This too, shows that quails were least susceptible to the toxic effects of diclofenac among the three species evaluated. Elevations of serum uric acid and creatinine concentrations have been observed in response to NSAID therapy in humans (Caspi et al., 2000; Koseki et al., 2001), Gyps vulture (Oaks et al., 2004; Swan et al., 2006), domestic fowl (Naidoo et al., 2007), and broiler chickens (Reddy et al., 2006). These results are partially supported by the findings of another study in which no changes in blood uric acid level were observed in bobwhite quails treated with flunixin meglumine (Klein et al., 1994). Likewise, Pereira and Werther (2007) found no change in the blood uric acid concentrations in budgerigars treated with different NSAIDs. The difference in susceptibility to uric acid elevations after exposure to different NSAIDs seems to be related to variations in bird species. In the current experiment it was observed that quails were least affected with the toxic effects of diclofenac. This observation is in complete agreement with that reported by Lucas (2003), who found no renal impairment in Japanese quails treated with flunixin or meloxicam.

Different NSAIDs were screened for their toxicity profiles in broiler chickens to find safer alternatives than diclofenac. Metamizole, phenylbutazone, ketoprofen, meloxicam, piroxicam and flunixin were evaluated in this experiment. Birds in the various groups exhibited no clinical signs except in the flunixin group. The clinical signs exhibited by the birds in this group were identical with those observed in the birds that experienced diclofenac toxicosis in previous experiments. The clinical signs
associated with the toxicity of these drugs are considered to be largely a result of kidney damage. Similar clinical signs were observed in vultures (Oaks et al., 2004; Shultz et al., 2004) and fowl (Naidoo et al., 2007) given diclofenac that developed renal failure. Although, pH of the blood was not measured in this study, it is reported that the development of acidosis due increase in blood uric acid might lead to the signs of depression as observed in mammals (Speer, 1997). It has been reported that hyperkalemia which occurs as a result of renal failure can lead to cardiac arrest and ultimately death (Lumeij, 1994). The chickens treated with diclofenac and flunixin showed similar signs as described in the above-mentioned studies. The absence of clinical signs in chickens treated with phenylbutazone, dipyrone, ketoprofen, miloxicam and piroxicam correlated with the lack of damage to the kidney tissues in these groups. No abnormal concentrations of serum uric acid or creatinine and no microscopic lesions in kidney tissues were recorded in these groups.

Phenylbutazone and dipyrone have been used for the treatment of pyrexia, various types of colic, and arthritic conditions including, osteoarthritis and ankylosing spondylitis. The use of these drugs is limited especially in dairy cattle due to concerns regarding potentially serious adverse effects, specifically blood dyscrasias, agranulocytosis, aplastic anemia and mutagenicity, in humans who consume residues in dairy products (FDA, 2003; Izumi et al., 1991; Watson et al., 1980; Weiss & Klausner, 1990). The newer NSAIDs like flunixin meglumine and diclofenac are very effective anti-inflammatory, analgesic and antipyretic drugs (Amiridis et al., 2001; Anderson et al., 1986a; Barnett et al., 2003; Swinkels et al., 1994). Both of these drugs are reported to cause renal damage and visceral gout leading to death in bird species (Klein et al., 1994; Oaks et al., 2004; Shultz et al. 2004). The results of present study are in line with these research investigations. In our study it was found that
ketoprofen, meloxicam and piroxicam are effective in reducing inflammation, lowering pyrexia and alleviating pain in buffalo and horses. Similar observations have been reported by several researchers (Banting et al., 2008; Bressan and Tonussi, 2008; Doig et al., 2000; Faulkner & Weary, 2000; Hirsch et al., 2003; Mathews et al., 2001; Richy et al., 2009)
Chapter 6

SUMMARY

A catastrophic decline in vulture populations was first observed in 1996-97 in Keoladeo National Park, Bharatpur, Rajasthan, India. Later, similar situations were reported in many south Asian countries including Pakistan, Nepal and Bangladesh. Now, the International Union for the Conservation of Nature (IUCN) has listed three vulture species i.e. *Gyps bengalensis*, *Gyps indicus* and *Gyps tenuirostris* as ‘critically endangered’.

Vultures are natural scavengers and play a key role in keeping the environment clean by consuming carcasses of dead livestock and wildlife. The unconsumed animal carcasses pose a serious threat to both human and animal health because decaying animal carcasses may contaminate groundwater and become a potential source of diseases such as tuberculosis and anthrax for humans and other animals. Also, due to the declines in vulture populations, a rise in the numbers of other scavengers like dogs has been observed. Therefore, the risk of dog bite and transmission of dangerous diseases, including rabies, has also increased. Furthermore, vultures play a vital role in the disposal of human corpses of the followers of the Parsi religion because they place their dead bodies before vultures for disposal rather than burial beneath the earth.

In the South Asian region, different communities have different attitudes toward the use of meat and its products. For example, Muslims do not use meat of dead animals, whereas Hindus abstain from cow’s meat altogether because of religious bindings. For many centuries, the disposal of such meat has been done by vultures.

The vulture population decline issue was investigated by researchers with the support of various international organizations dedicated to bird conservation in collaboration with
regional ornithological societies. Scientists looked into the problem from different angles and considered a variety of reasons of this decline. These included food shortages, losses of habitat, persecution, human disturbances, infectious agents, environmental contaminants, intentional poisoning, and accidental poisoning through food or water. Initially, the outbreak of some infectious disease and/or poisoning appeared to be the most plausible basis of this crisis. Recent studies have ruled out presence of a widespread infection and have focused on some form of poisoning. Now, it is believed that diclofenac, a veterinary drug, was responsible for the huge fatalities in vultures, in part because the onset of the crisis was coincident with the introduction of this drug in veterinary practice.

Diclofenac belongs to a class of drugs called, non-steroidal anti-inflammatory drugs (NSAIDs). Diclofenac served as an effective analgesic (pain killer), antipyretic (reduces fever), and anti-inflammatory (reduces swelling) drug. Initially, this drug was used in human beings for various indications such as arthritis. The use of diclofenac was started in domestic animals in the region a decade ago. It was reported that the presence of diclofenac in the bodies of dead animals that had been treated with this drug shortly before death was harming the vultures feeding on contaminated carcasses (Oaks et al., 2004; Shultz et al., 2004; Swan et al., 2005).

The Department of Pharmacology and Toxicology, at the University of Veterinary and Animal Sciences, Lahore, has been given the task to investigate this problem. A series of experiments was conducted to study the likely causes of this problem and, if possible, find its solution by developing safer and efficacious alternatives to diclofenac for the treatment of animals. For this purpose, a chicken experimental model was developed to study the toxicity of diclofenac and other NSAIDs. These studies have shown that diclofenac produced similar
toxic effects and mortalities in broiler chickens as had been reported from studies of diclofenac-poisoned vultures. Later on, therapeutic efficacy studies of safer alternative NSAIDs of diclofenac were conducted in horses, buffaloes.

On the basis of these current studies, it was concluded that diclofenac was toxic to chickens and no significant difference was present in the death rates in bird groups treated with toxic doses of diclofenac via oral and intramuscular routes. Sodium and potassium salts of diclofenac caused comparable casualties in broiler chickens (unpublished study). A number of other NSAIDs were screened for their toxicity profile using the chicken model. This study showed that phenylbutazone, dipyro, meloxicam, piroxicam and ketoprofen were comparatively safer than diclofenac. However, phenylbutazone and dipyro may not be appropriate alternatives for diclofenac in food-producing animals because they are known to cause a condition called agranulocytosis in human and animals. Agranulocytosis involves marked reductions in numbers of white blood cells that are responsible for maintaining the body’s immunity against various diseases.

To evaluate the efficacy of safer drugs, fever was induced in buffalo calves with *Escherichia coli* endotoxin (lipopolysaccharide), and the animals were then treated them with ketoprofen, meloxicam or piroxicam. Although, all three drugs were effective in lowering body temperature, ketoprofen was the most efficient. In another experiment, these drugs were used in the treatment of lameness in horses, and it was concluded that meloxicam was more effective followed by piroxicam and ketoprofen for the treatment of this particular problem.

Based on these observations, it is concluded that ketoprofen, meloxicam and piroxicam may prove quite safe drugs for the scavenging birds and may be used as safe alternatives to diclofenac in veterinary practice. It has been observed during this study that ketoprofen,
piroxicam, and meloxicam are less toxic for broiler chickens and may prove better alternates to be used in place of diclofenac in animals. The use of these NSAIDs may be less toxic for scavenger birds. The commercial preparations of ketoprofen and meloxicam for veterinary use are available at least in two south-east Asian countries i.e. Pakistan and India. The prices of these NSAIDs are comparable with those of the veterinary preparations of diclofenac which were previously available in the region. On the basis of results of this study it is concluded that ketoprofen, piroxicam, and meloxicam are effective NSAIDs in domestic animals, hence may replace diclofenac in veterinary practice.
Chapter 7

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