Protective Effects of Allopurinol on Diclofenac-Induced Toxicity in Domestic Chicken

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ABSTRACT

Diclofenac, a non-steroidal anti-inflammatory drug is becoming a threat to ecological balance by its swipe at vultures. The decline in population of vultures has been attributed to high mortality due to diclofenac toxicity, which occurred via ingestion of carcasses treated with diclofenac. The objective of this study was to establish the effect of probenecid and allopurinol in preventing gout with urates in diclofenac-induced toxicity in domestic chickens. Dominant brown pullets from Zartech hatchery in Benin City were selected for this study. Four groups of five layers each were used for the various tests. Findings from our study showed that signs of diclofenac toxicity were severe in all test groups except for the group treated with allopurinol where mortality was reduced and survivors were more than observed in the other groups. The gross pathological lesions at necropsy were mild when compared with lesions observed in the other test groups. One observation from this study among birds treated with diclofenac only was that there were survivors despite the fact that a lethal dose of 10mg/kg of the drug was administered to all the chickens used in this study. That there were survivors establishes the fact that diclofenac toxicity is not uniformly lethal once clinical signs develop. Among birds treated with probenecid, gross pathological lesions observed at necropsy were a lot more severe than other groups hence probenecid is not suitable for the avian species since it did not remedy nor prevent diclofenac toxicity, rather it made it worst.

Key words: Diclofenac, Allopurinol, Toxicity, Probenecid, Chickens

INTRODUCTION

Diclofenac, a non-steroidal anti-inflammatory drug is turning out to be a threat to ecological balance by its swipe at vultures, nature’s scavengers[1]. Diclofenac toxicity has been associated with the noticeable decline of Oriental white-backed vulture (Gyps bengalensis), long-billed vulture (Gyps indicus) and slender-billed vulture (Gyps tenuirostris) population which has crashed in the last decade in India and Pakistan [1]. This decline has been attributed to high mortality due to diclofenac toxicity, which has occurred in these birds by ingesting the carcasses of animals treated with diclofenac prior to death [2]. Evidence suggests that vultures which fed on the carcasses of cattle treated with diclofenac to reduce pain, eventually died of visceral gout and renal failure. This has resulted in a drastic decline in the number of vultures in India and Pakistan and its negative ecological consequences one of which is a threat of epidemics because of decaying and unscavenged carcasses [3]. Diclofenac has been reported to cause the death of Oriental white-backed vultures that ingested carcasses of domestic livestock and at postmortem the prominent lesions in the said vultures were severe acute necrosis of the proximal convoluted tubules [4]. This toxicity has since been reproduced under controlled experimental conditions in both captive Oriental and African white-backed vultures, and diclofenac was found to cause similar clinical signs in all the vultures. The birds became depressed at approximately twenty-four hours after exposure and this downward decline progressed until they died 36-48hr post exposure [5]. Similar signs have also been described in the field where sick birds were observed to be very depressed in their nests and fall over dead [6]. Vultures experimentally exposed to diclofenac were also reported to have developed visceral gout and died of acute renal necrosis [4, 5]. Only one report described an experimental comparative toxicity study using Nimesulide and Diclofenac Sodium in chickens [7]. In another study, investigators attempted to validate the domestic fowl as a model, to allow for the further characterization of diclofenac’s toxicity.
Acute toxicity of Diclofenac Sodium in chickens

Experimental Plan

A total of 20 birds, five represented a group. There were 4 groups making up the study because of availability. The birds were reared by Zartech hatchery in Benin City, Nigeria and reared until they were 19 weeks of age. The chicken was administered to individual birds intramuscularly at a lethal dose of 10mg/kg. The birds were weighed individually and the appropriate amount of drug was calculated for each bird. A single injection of diclofenac sodium was administered to the right caudal 1/3rd of the pectoral muscle of the test chickens at a dose of 10mg/kg.

Clinical Examination: The chickens were subjected to clinical examination twice daily (morning and evening) during the study. Clinical examination included general appearance and behaviour of the birds, response to external stimuli (hand clapping), eating and drinking as well as colour and consistency of faeces.

Haematological Examination: Blood samples were taken via venipuncture from the wing vein (v. cutanea ulnaris) 24hours after drug administration and transferred immediately to a 2 ml glass tube containing ethylenediaminetetraacetic acid (EDTA) for haematological analyses. Thin smears were made immediately and haematological analyses were performed as described by Jain (1986). Total red blood cells (RBC) and total white blood cells (WBC) counts were performed by a manual method using haemocytometer with blood diluted on 0.01% of toluidine blue stain. The haemoglobin concentration (Hb conc) was measured by cyanmethaemoglobin method. The Packed Cell Volume (PCV) was measured by micro haematocrit capillary tubes, centrifuged at 2500 rpm for 5 min. The differential WBC counts were made on blood films stained with Wright’s stain, using an average of 200 cells. The heterophil/lymphocyte ratio was determined by division of number of heterophils by the number of lymphocytes. The total plasma protein was determined by the refractometer using the method described by Jain (1986).

Serum Chemistry: Blood samples were collected aseptically by venipuncture from the wing vein and transferred to a 10 ml plain glass tube containing no anticoagulant for serum chemistry analysis. Blood samples were kept in a slanted position in an ice-pack. Clear sera were collected in sterile vials, labelled and stored in a freezer at -20°C until analysis. Serum chemistry was done at the Central Diagnostic Laboratory, National Veterinary Research Institute Vom, Jos, Plateau State, Nigeria. The electrolytes Na⁺, K⁺ and Cl⁻ were measured as well as the uric acid and creatinine. The liver enzymes ALT, AST and ALP were also determined at the same laboratory.

Pathomorphological Examination: Immediately after death, postmortem was done on dead birds and lesions observed grossly were recorded. Tissue samples were collected and these included parts of the liver, kidney, lungs and heart. These samples were submitted for histopathological examination. The tissues were preserved in 10% buffered formalsaline before being taken to the laboratory (NVRI, Vom). At the laboratory...
they were trimmed and prepared routinely for histopathology using the hematoxylin and eosin stains. Birds from the control group were subjected to the

Table 1: Haematological Parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Test A (Diclofenac)</th>
<th>Test B (Diclofenac + Allopurinol)</th>
<th>Test C (Diclofenac + Probenecid)</th>
<th>Group D (Control)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>4.7±(0.17)</td>
<td>4.4±(0.52)</td>
<td>3.1±(1.00)</td>
<td>4.6±(0.78)</td>
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<td>Platelet count</td>
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<td>6.7±(1.06)</td>
<td>2.4±(0.21)</td>
<td>5.1±(1.61)</td>
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<td>WBC</td>
<td>16.7±(2.95)</td>
<td>23.9±(8.95)</td>
<td>20.3±(7.32)</td>
<td>13.9±(2.00)</td>
<td>&lt;0.01</td>
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<td>PCV</td>
<td>20.8±(0.84)</td>
<td>19.4±(2.30)</td>
<td>19.2±(2.17)</td>
<td>25.4±(3.65)</td>
<td>&lt;0.01</td>
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<td>Hb conc.</td>
<td>16.8±(0.94)</td>
<td>13.3±(0.23)</td>
<td>12.6±(0.10)</td>
<td>15.3±(0.78)</td>
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<td>Lymphocyte count</td>
<td>86.0±(5.43)</td>
<td>93.2±(3.56)</td>
<td>85.8±(13.81)</td>
<td>77.6±(8.26)</td>
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<tr>
<td>Leukocyte count</td>
<td>6.6±(0.55)</td>
<td>7.8±(3.03)</td>
<td>18.6±(15.50)</td>
<td>16.0±(7.48)</td>
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Mean values with different superscript differ significantly while Mean values with same superscript differ slightly. Data was subjected to one way analysis of variance (ANOVA). Variation among means (M) was significantly greater than expected by chance.

Table 2: Serum Chemistry

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Test A</th>
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<th>Test C</th>
<th>Control</th>
<th>P value</th>
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<tr>
<td>Liver Function Tests</td>
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<td>ALT</td>
<td>ALP</td>
<td>Serum</td>
<td>Creatinine</td>
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<td>21.9±(5.79)</td>
<td>37.5±(3.68)</td>
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<td>34.1±(1.43)</td>
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<td>18.5±(1.36)</td>
<td>38.5±(2.09)</td>
<td>41.4±(1.67)</td>
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<td>21.8±(2.60)</td>
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<td>47.0±(6.93)</td>
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<td>Kidney Function Tests</td>
<td>Uric Acid</td>
<td>Serum Na⁺</td>
<td>Serum K⁺</td>
<td>Serum Cl⁻</td>
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<tr>
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<td>236.6±(36.43)</td>
<td>7.32±(0.90)</td>
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<td>171.8±(2.59)</td>
<td>185.0±(23.40)</td>
<td>8.6±(0.57)</td>
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<td>9.7±(1.43)</td>
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<td>4.8±(1.11)</td>
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<td>4.8±(1.11)</td>
<td>108.2±(5.54)</td>
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*Mean values with different superscript differ significantly while Mean values with same superscript differ slightly. Data was subjected to one way analysis of variance (ANOVA). Variation among means (M) was significantly greater than expected by chance.

Fig 1: (A) a - Avian heart enveloped with urate deposits, b - Hepatic necrosis  (B) Cross section of the avian visceral organs of a chicken treated with Allopurinol appears grossly normal (C) Cross section of the visceral organs covered with uric acid crystals in a chicken treated with Probenecid.
same examination and sacrificed at the end of the study.

Effect of Allopurinol on Diclofenac-induced toxicity in Chickens (Group B): Five layers at the beginning of their production cycle were given allopurinol per os. The drug was dissolved in 0.1N NaOH and administered directly into the crop at a dose of 25mg/kg, a safe dose for chickens SID \[10\]. One hour later, a single injection of diclofenac sodium was given to the test chickens at a dose of 10mg/kg via the right caudal 1/3rd of the pectoral muscle.

Effect of Probenecid on Diclofenac-induced toxicity in Chickens (Group C): Five layers at the beginning of their production cycle were given probenecid orally in
Effect of Allopurinol on Diclofenac-induced toxicity in Chickens:

On day 4, two of the birds showed signs of toxicity: birds appeared to be asleep and had stopped eating. They responded to external stimuli by clapping but after few minutes became depressed again. This depression on the average occurred 24 hours post-dosing and progressively became more severe for all the birds. Prior to death the affected chickens appeared to be in coma and eventually died. Mortalities were recorded on day 2 and 3. There were no survivors as 100% mortality was recorded in this group of birds.

RESULTS

Clinical Signs: Acute toxicity of Diclofenac Sodium in Chickens

In this group, the birds were severely depressed, reluctant to move, perched in one corner of their cages, appeared to be asleep and had stopped eating. Birds initially responded to external stimuli (hand clapping) by waking up but within a few minutes became depressed again. Depression on the average occurred 24 hours post-dosing and progressively became more severe. Prior to death the affected chickens appeared to be in coma and could not be aroused by any amount of noise. On day 1, there was no mortality recorded, however, one death and two deaths were recorded on day 2 and 3 respectively giving a total of three mortalities and two survivors by the end of the third day. The surviving birds came to lay at the 24th week of life and the eggs were well formed with ratio of shell weight: thickness remaining constant.

Effect of Allopurinol on Diclofenac-induced toxicity in Chickens:

In this group after treatment with allopurinol and subsequently with diclofenac, clinical signs observed were: birds were quite alert, moved around their cages, appeared to be active and eating very well. Birds responded to external stimuli by the first and second day however became gradually depressed by the third day post-dosing. This depression on the average occurred 48 hours post-dosing and progressively became more severe for two of the birds. Prior to death the affected chickens appeared to be sleeping and eventually died. Mortality of two birds was recorded on day 4 with three survivors, which by the end of the third day showed no signs of toxicity. The surviving chickens however had a slight delay in egg production coming to lay by the 21st week of life.

Effect of Probenecid on Diclofenac-induced toxicity in Chickens:

In this group, after treatment, birds were quite alert, moved around their cages, appeared to be active and eating well. They responded to external stimuli by the first day but however gradually became depressed (dull and sleepy) by the second day post-dosing. The birds stopped eating and responded to hand clapping but after few minutes became depressed again. This depression on the average occurred 24 hours post-dosing and progressively became more severe for all the birds. Prior to death the affected chickens appeared to be in coma and eventually died. Mortalities were recorded on day 2 and 3. There were no survivors as 100% mortality was recorded in this group of birds.

Haematological Examination: Changes in RBC, platelet count, WBC, PCV, Hb concentration and lymphocyte count in the blood of investigated chickens are presented in Table 1. Significant changes in the RBC (million/µl) were observed in test group C (probenecid + diclofenac) when compared with those in the control group. Changes in platelet count (million/L) in peripheral circulation were observed in test group A (diclofenac only) and test group C. Significant changes in WBC (thousand/µl) in peripheral circulation were observed in birds in all the treatment groups while lymphocyte count (%) and heterophil leukocyte count increased significantly in all the test groups when compared with the control group (Table 1).

Serum Chemistry: Findings of both liver function tests and kidney function tests are presented in Table 2. Serum aspartate aminotransferase (AST) activities were determined and differed significantly in the test groups when compared with the control group except for those treated with diclofenac only. Serum alanine aminotransferase (ALT) activity differ slightly in the test groups when compared with the control group except for birds in test group C. Serum alkaline phosphatase (ALP) activity and serum creatinine levels (mg/dl) differ significantly in the test groups when compared with the control group. Serum uric acid level (mmol/L) differ slightly in the test groups when compared with the control group except for those in test groups A and C which were in the same range as the control group. Serum sodium, potassium and chloride levels (mmol/L) differ significantly in the test groups when compared with the control group.

Pathomorphological Examination: Gross Pathology:

Severe visceral gout affecting the liver and kidney was evident in all the birds that died. There was necrosis of the kidney due to apparent change in consistency. In the birds necropsied within minutes of dying, the
pericardium and pericardial fluid was white, chalky and opaque covering the epicardium and attached to it. The liver was friable. In chickens treated with probenecid, gross examination of the internal organs revealed that 2/3rd of the liver, air sacs, heart and lungs were covered with urate crystals though these organs were observed to adhere to the wall of the pectoral muscle. The kidneys were two times larger with rounded edges when compared to the normal kidneys of birds from the control group, the liver was friable, there was froth in the pleural cavity and haemorrhages in the caecal tonsil. The heart was completely enveloped with a cheesy white layer (Figure 1A). Grossly normal visceral organs were seen in chicken treated with allopurinol (Figure 1B) while chickens treated with probenecid had urate deposits (Figure 1C).

Histopathology
The effect of allopurinol on diclofenac-induced toxicity in chickens resulted in moderate congestion and haemorrhages in the kidneys. The cortical collecting ducts had their lining cells dislodged from their basement membrane and some sloughed into the lumen while some were misshapen. There was moderate congestion and hemorrhage (Figure 2A). The parabronchi of the lungs were collapsed and there was expansion of the air capillaries as well as moderate haemorrhage into the lung tissue which extended into the air capillaries. In the liver, there was moderate hepatic necrosis, and fragmentation of hepatocytes.

The effect of probenecid on diclofenac-induced toxicity in chickens resulted in massive coagulative necrosis of all resident cells/tissues of the kidney with vacuolation. There was severe congestion and haemorrhage with numerous eosinophils in the medullary ducts. Some necrotic tubules had glassy radiating appearance (figure 2B) and there was expansion of the Bowman’s space. There were generalized multiple foci of necrosis with some areas vacuolated. In the liver, mild haemorrhage and congestion of the hepatic vessels were observed with very mild periportal heterophilic infiltration (figure 2C). There was generalized necrosis and individualization of hepatocytes (figure 2D). The capsule was damaged and there were few karyorrhetic cells in this region. There was congestion with sinusoidal expansion. The lungs of affected birds showed severe congestion and haemorrhage of the lung tissue which extended into the air capillaries and parabronchi. There was collapse of the parabronchi and expansion of some air capillaries (figure 2E). There were foci of necrosis affecting the air and blood capillaries with a glassy appearance. In the heart, there was a focus (peri-myocardial) with slight inflammation (macrophages, heterophils and lymphocytes were present).

DISCUSSION
The clinical signs observed in birds given diclofenac only (group A) include: sitting on the hock with eyes closed, reduced feed and water intake, depression, somnolence and lethargy. These signs are similar to those reported for G. africanus [5] and the domestic fowl [3]. Another report also shows that severity of clinical illness and mortality at given dose levels differ between species [12]. In trying to remedy the acute toxicity caused by diclofenac, two groups of chicken were treated with allopurinol and probenecid respectively. From this study, it was observed that all test groups showed clinical signs of diclofenac toxicity and some of the birds eventually died. In the allopurinol treated group, depression set in 48h post dosing with diclofenac and 40% mortality was recorded. In the group treated with probenecid, depression occurred 24h post dosing with diclofenac and the clinical signs of toxicity were severe resulting in 100% mortality. The mortality rate of 40% in group (B) in this study is similar to 33% mortality reported by Naidoo et al. (2007) in Leghorn layers and 40% described by Reddy et al. (2006) in chickens. However the mortality rate of 40 % in group (B) is lower than that observed in another study conducted on Oriental white-backed vulture (Gyps bengalensis) dosed 2.5mg/kg of diclofenac in which the mortality rate was 100% [2] and this may suggest that vultures are more susceptible to diclofenac toxicity which corroborate earlier findings [12].

In the diclofenac-treated group, ALT differed slightly statistically when compared with parameters for the control birds. Swan et al (2006) mentioned an elevation in serum ALT in vultures following diclofenac administration [5]. In birds treated with allopurinol, AST was statistically significant while ALT differed slightly when compared with those in the control in contrast to the picture in typical diclofenac toxicity. However birds treated with probenecid, had statistically significant difference in AST and ALP when compared with the control group. In the diclofenac-treated birds, the serum creatinine levels were statistically significant. Hussain et al (2008) reported that serum creatinine level had a regular and uniform pattern in all species being significantly elevated in diclofenac-treated groups. Elevation of serum urea and creatinine levels are indicators of nephrotoxicity [16]. Serum Na+ and K+ levels were significantly higher than the control group in diclofenac-treated birds. It has been reported that there is a terminal increase in potassium concentrations in the dead fowl’s plasma [3] and it has also been attributed as the cause of death in gouty fowls [10]. This is similar to the findings in this study especially with
the birds that were treated with probenecid. In the allopurinol-treated group, serum creatinine level, serum uric acid level and serum Cl- level differed significantly when compared with those in the control group. While the serum Na⁺ and K⁺ levels were significantly higher than that recorded for the control group.

Grossly in all the treatment groups, most visceral organs showed deposition of uric acid crystals or gout recognized as a white crystalline powder present on the visceral surfaces of these organs. The pathological changes observed in the chickens following diclofenac treatment was similar to that reported in the Gryps species i.e diffuse visceral gout to severe nephrosis [2,4,5]. In the allopurinol treated group the visceral gout was not so pronounced and the nephrosis was mild. The visceral organs did not adhere to the wall of the pectoral muscle like in the other test groups. There was severe and acute pathology of several organs in chickens that died from diclofenac toxicity treated with probenecid. However the extent of damage varied among birds. Visceral organs were observed to adhere to the wall of the pectoral muscles. The kidneys were enlarged, the liver was friable and there was froth in the pleural cavity. The heart was completely enveloped with a cheesy white layer. A similar finding in diclofenac-treated white leghorn layers had also been reported [3]. The heart and liver were observed to have adhered to the wall of the pectoral muscle with fluid present in the peritoneal cavity. There was haemorrhage in the caecal tonsil as well as along the length of the gastrointestinal tract. This finding has however not been documented in previous related studies.

At necropsy, birds treated with diclofenac showed severe and generalized multiple foci of renal necrosis characterized by massive urate deposition on visceral organs similar to other reports. There were also concentric areas filled with necrotic debris and hardly any normal renal tissue present at histopathology and consistent with previous studies [2,4,5]. Kidneys of birds in group B showed moderate congestion and haemorrhage, similar to that described in other avian species with diclofenac toxicity [2,4,5]. The mechanism of urate accumulation on and within visceral organs as observed in birds in the probenecid-treated group is believed to be due to an initial diclofenac metabolites-induced kidney dysfunction. The kidney dysfunction results in impaired excretion of uric acid leading to progressive hyperuricemia and consequently the characteristic deposition of monosodium urate crystals [17].

Liver congestion, haemorrhages, fatty change and necrosis of hepatocytes were consistent findings in birds treated with diclofenac [12,16], which is consistent with the findings in this report. In the allopurinol-treated birds, there was moderate hepatic necrosis and fragmentation of hepatocytes. In the probenecid-treated birds, the liver exhibited mild haemorrhage and congestion of the hepatic vessels was observed with very mild periportal heterophilic infiltration. Reddy et al. (2006) described lesions in kidneys of diclofenac-treated chickens and similar lesions were observed in this study [7]. However they did not report aggregates of uric acid crystals or gout in the kidneys. Gross and histopathological lesions in the kidneys of these chickens revealed acute tubular necrosis. Similar kidney lesions have been observed in vultures suspected to have died of diclofenac toxicity under field conditions [3] as well as those given diclofenac under experimental conditions [4,5]. The exact mechanism of nephrotoxicity induced by diclofenac is not known. However, diclofenac is metabolized in the liver, principally into 4-hydroxy diclofenac along with other hydroxylated metabolites. These metabolites are excreted through the kidney (65%) and bile (35%) [19].

The clinical signs of severe depression, coma and death in the chickens as well as in G. africanus vultures in previous studies, appear to be directly related to the increase in uric acid in the plasma and hyperkalaemia [3]. The visceral gout observed in the diclofenac-treated chickens in this study was a consistent feature in vultures which died from natural or experimental diclofenac toxicity [2,4,5]. The presence of uric acid crystals in the visceral organs reported in diclofenac toxicity in vultures by the authors above were also observed in kidneys of the chickens used in this study. Limitation of the study was that haematological parameters were not taken on birds prior to injecting them with drugs due to cost of running the tests but control group haematological values were deemed to adequately represent the general normal values.

In this study, we tried to prevent diclofenac toxicity by treating the chickens with two drugs; allopurinol and probenecid. In conclusion this experiment showed that signs of diclofenac toxicity were severe in all the test groups except for the group of chickens treated with allopurinol, a xanthine oxidase inhibitor (test group B). In group (B), mortality was reduced and the survivors were more than the number recorded in other test groups. The gross pathological lesions at necropsy were mild when compared with those observed in the other test groups. In the group given diclofenac alone there were survivors (40%) as well which was unexpected since the lethal dose of 10mg/kg of the drug was administered to all the chickens used in this study, this however is consistent with previous findings that diclofenac toxicity is not uniformly lethal once clinical signs develop [3]. One may conclude also that probenecid, a uricosuric agent is not ideal for avian species since it did not remedy or prevent diclofenac...
toxicity, rather it made it worse. The gross pathological lesions observed at necropsy were a lot more severe than were recorded in all the other groups in this experimental study.

ACKNOWLEDGEMENT
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Authors Contribution: E.I.K.O and M.K.A developed the concept and designed experiments. M.K.A carried out the experiments. M.K.A and O.A analyzed the data. E.T.I carried out the histopathology and read the slides. M.K.A, E.T.I, O.A contributed to the manuscript write up and E.K.I.O summarized and finalized the manuscript.

REFERENCES