



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

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

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6.1 Abstract

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

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

Key Words

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



6.2 Introduction

In the recent past the dramatic decline in vulture numbers across the Indian subcontinent has illustrated the impact a simple chemical compound could have in the environment (Prakash *et al.*, 2003, Green *et al.*, 2004, Oaks *et al.*, 2004). Populations of Oriental white-backed vultures (*Gyps bengalensis*), long-billed vultures (*G. indicus*) and slender-billed vultures (*G. tenuirostris*) in the region have markedly decreased by more than 98% following exposure to the non-steroidal anti-inflammatory drug (NSAID) diclofenac over an estimated fifteen year period (Prakash *et al.*, 2003, Green *et al.*, 2004, Oaks *et al.*, 2004). At present the Oriental white-backed vulture has been the most affected of the species. This drug exposure resulted purely from drug residues within livestock carcasses left out to feed the vultures. The drug has an estimated LD₅₀ in the range of 0.098 to 0.225 mg/ kg (Swan *et al.*, 2006), which following exposure results in death within a few days. All the dead birds showed signs of renal failure, liver injury and diffuse visceral gout on post-mortem examination (Oaks *et al.*, 2004, Swan *et al.*, 2006). In 2006 Swan and co-workers showed the African white-backed vulture (*G. africanus*) to be as susceptible to diclofenac as its oriental cousins (Swan *et al.*, 2006) and therefore an adequate model to evaluate the toxicity of diclofenac.

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



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

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

6.3 Materials and Method

6.3.1 Collection of blood samples

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

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



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

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



6.3.2 Hematology

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

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



6.3.3 Serum Chemistry

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



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

6.3.4 *Statistical Analysis*

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

6.4 **Results**

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

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



6.5 Discussion

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

6.5.1 Erythron

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

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

The Hct was also significantly higher than the PCV in all the birds. Although the exact cause of the difference is unknown, it is likely due to the anticoagulant used. Hematology samples were collected in K₃EDTA, which is known to induce an artifactual decrease in the PCV, while not affecting the Hct (Cott *et al.*, 2003). According to Abbot this decrease may be as high as 2-4% (Abbot, 2006).



6.5.2 *Leukon*

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

6.5.3 *Plasma proteins*

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

6.5.4 *Plasma Electrolytes*

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



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

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

6.5.5 *Enzymes*

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

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

6.5.6 *Urea and Uric acid*

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



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

6.6 Conclusion

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

6.7 Acknowledgements

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

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

Parameter	Mean	SD	Min	Max	Reference Interval	
Hb (g/L)	196.60	16.83	142.00	241.00	162.94	- 230.25
RBC (x 10 ¹² /L)	2.55	0.22	2.01	3.03	2.11	- 2.98
Hct (L/L)	0.50	0.04	0.38	0.60	0.42	- 0.58
MCV (fL)	196.70	5.55	182.00	211.00	185.60	- 207.81
MCHC (g/dL)	39.3	1.53	35.40	42.00	36.25	- 42.35
WBC (x 10 ⁹ /L)	16.71	1.63	4.00	34.00	13.45	- 19.97
Hmat (x 10 ⁹ /L)	13.70	6.11	3.04	27.60	1.48	- 25.93
Himmat (Band) (x 10 ⁹ /L)	0.00	0.00	0.00	0.00	0.00	- 0.00
Lymph (x 10 ⁹ /L)	1.02	1.91	0.00	12.55	0.00	- 4.84
Mono (x 10 ⁹ /L)	1.57	1.04	0.10	5.78	0.00	- 3.65
Eos (x 10 ⁹ /L)	0.75	0.71	0.00	3.68	0.00	- 2.16
Bas (x 10 ⁹ /L)	0.00	0.00	0.00	0.00	0.00	- 0.00
PCV (%)	44.3	4.79	31.00	53.00	34.72	- 53.88



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

Parameter	Mean	SD	Min	Max	Reference Interval		
UA (mmol/L) [‡]	0.65	0.03	0.22	1.195	.058	-	0.724
Urea (mmol/L) [‡]	3.21	1.01	1.1	4.7	1.18	-	5.23
U:UA [‡]	5.47	1.39	2.2	12.7	1.39	-	2.69
Alb (g/L) ^{*†}	11.69	1.06	10.50	13.60	10.37	-	13.18
Globulin (g/L) [†]	21.78	1.14	16	29	16.76	-	28.30
ALT (U/L) ^{*†}	35.51	1.99	6.00	95.00	9.233	-	144.45
AST (U/L) [†]	1620.92	2.24	241	5120	238	-	6160
CK (U/L) ^{*†}	287.47	137.00	136.00	631.00	112.85	-	596.15

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



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

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

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



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

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



Diclofenac in Gyps Vultures: A molecular mechanism of toxicity

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Diclofenac (DF), a non-steroidal anti-inflammatory drug (NSAID) was shown to have caused a massive decline in the vulture populations on the Asian subcontinent¹⁻⁴. Although the use of diclofenac was recently banned, following conclusive proof supporting the safety of meloxicam, the toxic effect of other veterinary NSAIDs needs to be determined. One manner of determining a drug's safety would be to develop an *in vitro* model. In an attempt to establish this *in vitro* model it was first necessary to establish the mechanism of diclofenac's toxicity. For this purpose three hypotheses were proposed and tested: vasoconstriction of the renal portal veins with subsequent necrosis; increased production of reaction oxygen species (ROS) with subsequent activation of the mitochondrial apoptotic cycle; and a decrease in the secretion of uric acid by renal tubular epithelial (RTE) cells. To test these hypotheses *ex-vivo* tissue models were established using tissue from either the domestic chicken (*Gallus domesticus*) or the African White-backed vulture (*Gyps africanus*), both previously validated as models^{5,6}. We demonstrated that both diclofenac and meloxicam are directly toxic to the chicken and vulture renal tubular epithelial (RTE) cells following 48h of incubation. It was shown that toxicity was associated with an increased production of reactive oxygen species (ROS), which could be temporarily ameliorated by pre-incubation with uric acid (UA) due to its anti-oxidant activity. When cultures were incubated with either drug for only two hours, meloxicam showed no toxicity in contrast to the cellular toxicity present for diclofenac. In both cases no increase in ROS production was evident. In addition both drugs influenced the excretion of uric acid by interfering with p-amino-hippuric acid (PAH) channels. The effect on uric acid excretion persisted after the removal of the diclofenac but not meloxicam. We therefore concluded that vulture susceptibility to diclofenac toxicity resulted from a combination of an increase in cellular ROS, a depletion of intracellular uric acid concentration, an important anti-oxidant, and most importantly the pharmacokinetics profile of the drug in the plasma.

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The last decade has seen the catastrophic decline in the vulture population on the Asian subcontinent following their inadvertent exposure to diclofenac. Diclofenac toxicity in vultures being associated with only severe visceral gout on necropsy has led to the speculation that the kidneys are the site of drug toxicity⁷. Meteyer *et al.* proposed that the inhibition of renal prostaglandins (PG) and subsequent closure of the renal portal valves was a cause of renal ischemia¹⁸. Although current literature does not support this proposal as the closing of the renal portal valve in birds promotes renal perfusion it is still possible that diclofenac interferes with vascular tone of the renal portal vascular network^{8,9}. Ng *et al.* (2006) proposed that the DF toxicity results from ROS induced oxidative damage to the mitochondria and subsequent activation of the apoptotic cycle¹⁰. To test these hypotheses in avian models, organ baths with renal vasculature tissue or primary cell cultures, using tissue from the validated *G. domesticus* model, were set up. In all cases the toxicity of DF was compared to meloxicam (MLX) and related to the pharmacokinetics of each drug.

In addition to the above hypotheses, we further proposed that DF could be toxic due to the inhibition of UA excretion. Studies by Naidoo *et al.* (2007), demonstrated that the clinical signs of depression, following exposure to diclofenac, were a direct result of the marked increase in plasma uric acid concentrations⁵. Since the mammalian kidney is influenced by the uricosuric effects of DF and MLX through the inhibition of the basolateral UA-excreting organic anionic transporters (OATs) (also referred to as a PAH transporter), multi-drug resistance protein (MRP) and the apical UA reabsorptive channel known as URAT1, it is possible that a similar effect occurs in the vulture. This is supported by similar PAH and MRP channels that are involved with UA excretion in the chicken (avians do not reabsorb UA) as well as by both diclofenac and phenylbutazone being known to induce uricemia in a validated chicken model^{11,12 5,13-15}. To test this hypothesis an artificial renal tubule was set up using either chicken or vulture RTE cells. The results from the culture assay were confirmed *in vivo*.

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



indomethacin inhibited renal vasoconstriction by inhibiting contractile $PGf_2\alpha$ production via cyclooxygenase (COX) inhibition⁵. Both DF and MLX are COX inhibitors. The effect of COX inhibition lasting for up to 12h, will therefore explain the irreversible effect seen.

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

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

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

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



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

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

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

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



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

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



Methods Summary

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

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



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

For the transporter assay RTE cells, 500ul (10^6 cells/ml) were seeded onto the anipore membrane culture inserts, in 24 well microtitreplates and fed every three days¹⁹. After seven days of incubation, media was replaced with the HBSS transport medium with either PAH (chicken RTE) or UA (vulture RTE) as previously described^{20,21}. Total drug exposure was for 2 hours. The irreversible toxic effect of DF was established and evaluated 2 and 4h following drug removal (Initial drug exposure was 5h). Uric acid concentration was measured with a Nexet Chemistry Analyser (Alfa Klasserman, Bayer SA) while PAH concentrations was analysed as described²². For the *in vivo* change in UA stored serum samples (0, 0.15, 0.5, 1, 1.5 and 2h) from birds treated with intravenous DF (n=4) at 0.8 mg/kg (unpublished) and intramuscular MLX administration (n=4) at 2 mg/kg (Naidoo *et al.*, in press) from *G. corprotheres* were analysed.

7.1 Acknowledgements

This project was approved by the Animal Use and Care committee of the University of Pretoria. The project was funded by the Royal Society for the Protection of Birds (RSPB), The National Research Foundation of South Africa (NRF) and Bayer Animal Health South Africa. Vulture research was approved by Gauteng Nature Conservation and undertaken with the aid of the Lion and Rhino non-profit organisation.

7.2 Electronic Addendum

7.2.1 Methods

7.2.1.1 Chemical and Consumables

Diclofenac, Meloxicam, Penicillin, Streptomycin, DCFH-DA, Di-methyl sulphoxide (DMSO), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), trypsin, norepinephrine (NE), NaCl, KCl, MgSO₄, NaHCO₃, KH₂P0₄, CaCl₂, EDTA, glutamine, sodium L-lactate, sodium pyruvate, Glucose and HEPES, p-amino-hippuric acid (PAH) were obtained from Sigma South Africa. Debulco's modified Eagles's essential medium



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

7.2.1.2 Animals

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

7.2.1.3 Dose Extrapolations

For the organ bath studies, the concentration of diclofenac and meloxicam tested represent the maximum plasma concentration of these drugs in vultures. For the toxicity and transporter assay, the concentrations tested are based on the extrapolated LD₅₀ of the diclofenac/meloxicam in the chicken and vulture, depending on which cells were used

7.2.1.4 Organ bath studies

The cranial renal portal vein was dissected away from the parenchyma of the kidney. The vein was suspended in physiological saline solution (PSS) containing calcium. Contractile studies were undertaken as previously described²². The dissected vessels were attached onto the force transducer of a myograph (Danish Myo Technology). and were allowed to equilibrate for 60 minutes in oxygenated PSS (5% CO₂ in air). Initial contraction was stimulated with potassium chloride and thereafter with NE (10 µmol) before and after treatment with DF (0.42 µmol) or MLX (0.66 µmol). The reversibility of the contractile response to NE was determined by flushing out the organ chambers thoroughly at least three times, allowing the tissue to equilibrate prior to re-stimulation with NE, without exposing tissue to . DF was tested in quadruplicate and MLX in duplicate due to technical difficulties.



7.2.2 *In vitro* RTE assay

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

7.2.2.1 Establishing of Cell Cultures

From the chickens and the vulture, the kidneys were harvested immediately after euthanasia, homogenised with a scalpel blade and incubated overnight in DMEM with 1mg/ml of collagenase at 37 °C^{23,24}. The following morning the cell homogenate was passed through 250 and 38 micron filters to isolate the RTE cells²⁵. The isolated cells were washed three times at 200 x G with PBS and re-suspended in DMEM supplemented with 10% FCS, streptomycin and penicillin G²⁶.

7.2.2.2 Toxicity Assay

Chicken and vulture RTE cells, 200ul (10⁵ cells/ml) were seeded into 96-well plates²⁷. The assays were allowed to establish over 48 hours, prior to incubation with the drugs. The chicken and vulture RTE cells were exposed to various concentrations of DF and MLX based on plasma concentrations previously measured in these species. Following predetermined intervals, the cultures were incubated with MTT (0.44 umol) for five hours^{21,27}. The degree of formazan formation was determined by rinsing the wells with HBSS prior to the addition of 50µl of DMSO and subsequent reading on a Varion spectrophotometer at 570nm (1cm path length)²⁸. For the delayed toxic effect, chicken RTE cells were incubated with either DF or MLX for two hours (2 half-lives in the chicken), flushed with HBSS three times and replaced with fresh medium. Toxicity was evaluated as for the standard toxicity assay. In all cases the degree of cell death, following drug exposure, was evaluated as a percentage of growth of untreated wells on the same plate (% Cell Viability).



7.2.3 ROS studies

Chicken RTE cells were grown as for the toxicity assay. The cells were incubated with DF or MLX in the presence or absence of the uric acid for 6, 8 and 12h (Yu 1998). The cell cultures were thereafter incubated with DCFH-DA for 30 minutes and washed with HBSS²⁹. The change in absorption was read on a Verion spectrophotometer at 504 nm. The delayed toxic effect was determined using the same culture method as for the toxicity assay. The degree of ROS production was evaluated as a percentage of the ROS production in untreated cells grown on the same plate.



7.2.4 Transporter Assay

To ascertain if either DF or MLX had an influence on UA transport, stored serum samples following the intravenous DF (n=4) at 0.8 mg/kg and intramuscular MLX administration (n=4) at 2 mg/kg from *G. corprotheres* were analysed for the change in serum UA concentration in the first two hours following dosing⁶. Uric acid was measured with a Nexet Chemistry Analyser (Alfa Klasserman, Bayer SA).

To determine the *in vitro* influence on UA transport, 500µl of vulture RTE cells (10⁶ cells/ml) were seeded onto the anipore membrane culture inserts, in 24 well microplates and fed every three days. After seven days of incubation, the culture medium was replaced with the HBSS transport medium as previously described. The reaction was started by adding the drug into the basal well. Cell integrity was initially established by the addition of inulin into each well. For the standard assay the assays were exposed to various concentrations of DF and MLX following 2 hours of incubation to ascertain their effect on UA excretion. The assay was subsequently repeated with chicken RTE cells with PAH. PAH concentrations were analysed as previously described. Both the UA and PAH transporter assays were repeated in duplicate for each time point. The change in concentration in each well was established as the difference from the start to the stop of incubation. The net excretion was calculated as the change in the basolateral concentration



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

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

7.2.5 Statistics and Repeatability

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

7.3 References

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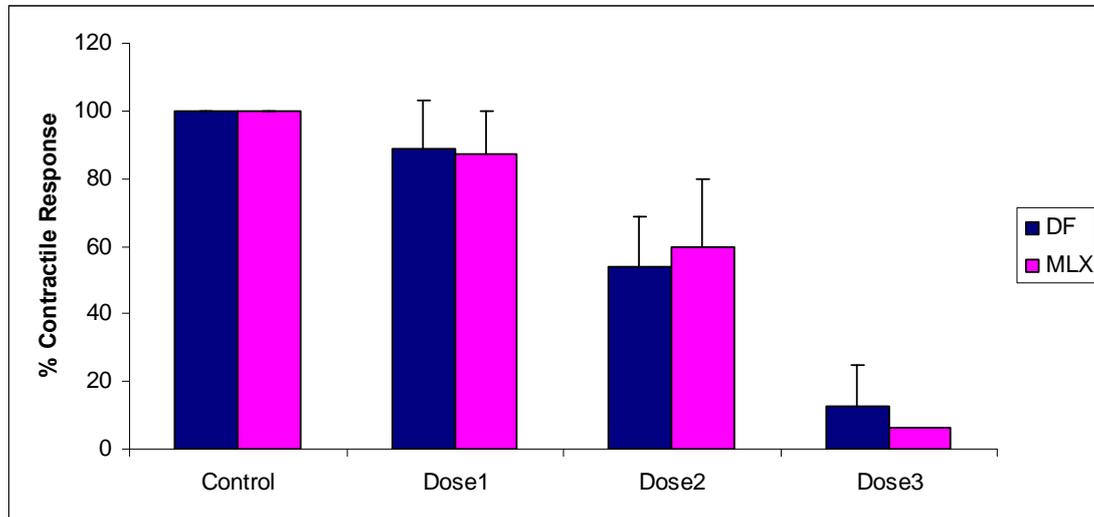


Figure 7-1: Contractile response of chicken cranial renal portal veins to norepinephrine (NE) alone (control) or the response to NE following co- incubated with either diclofenac (DF) or meloxicam (MLX) as a single dose of drug. Dose 1 to Dose 3 illustrates the additive inhibitory effect of the NSAIDs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

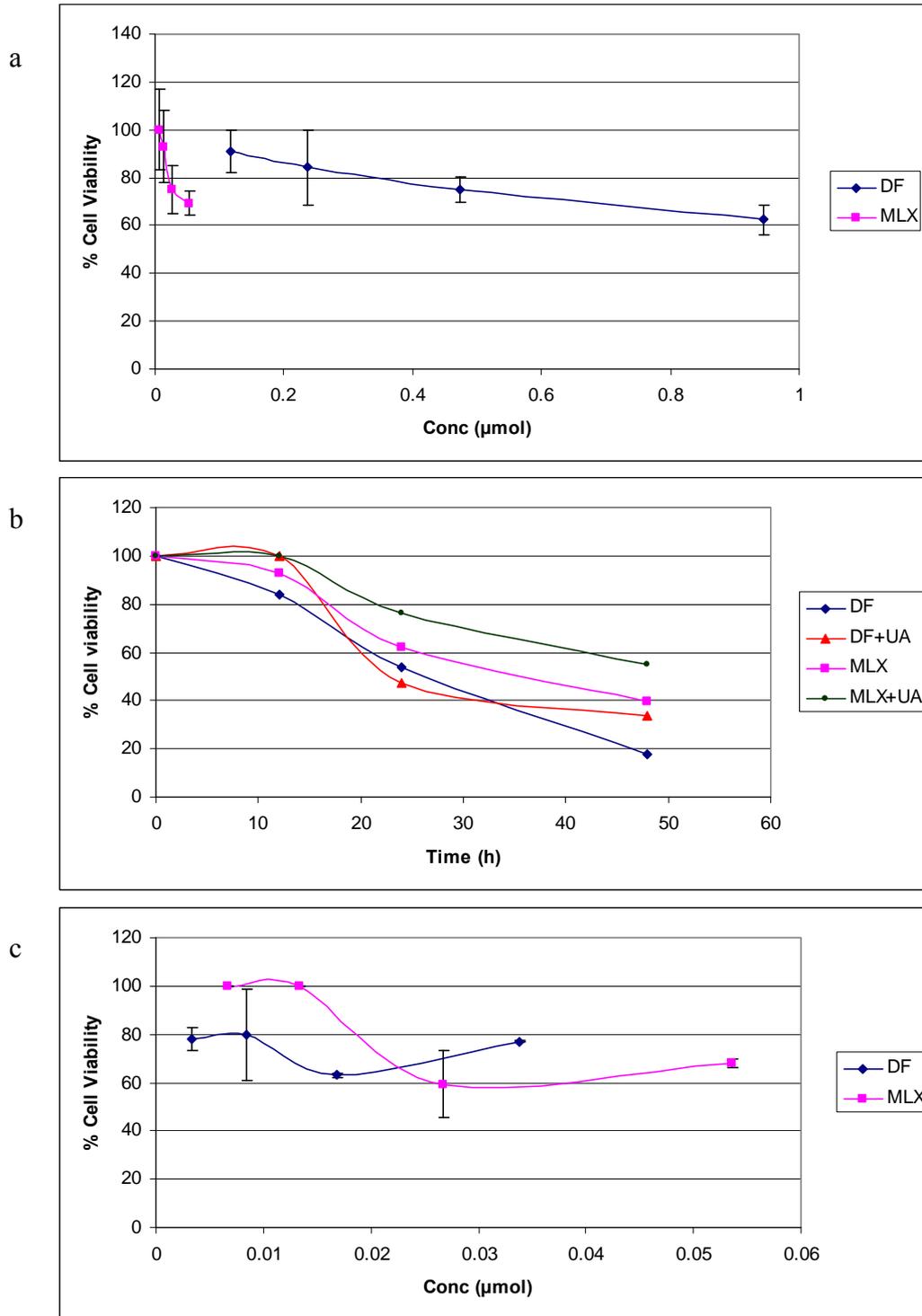


Figure 7-2: Direct cell toxicity of DF and MLX following variable periods of incubation and/or concentrations using the MTT assay. Results are presented as mean \pm SEM. a) chicken RTE cell viability following 12 hours of incubation with DF or MLX at four concentrations; b) chicken RTE cell viability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

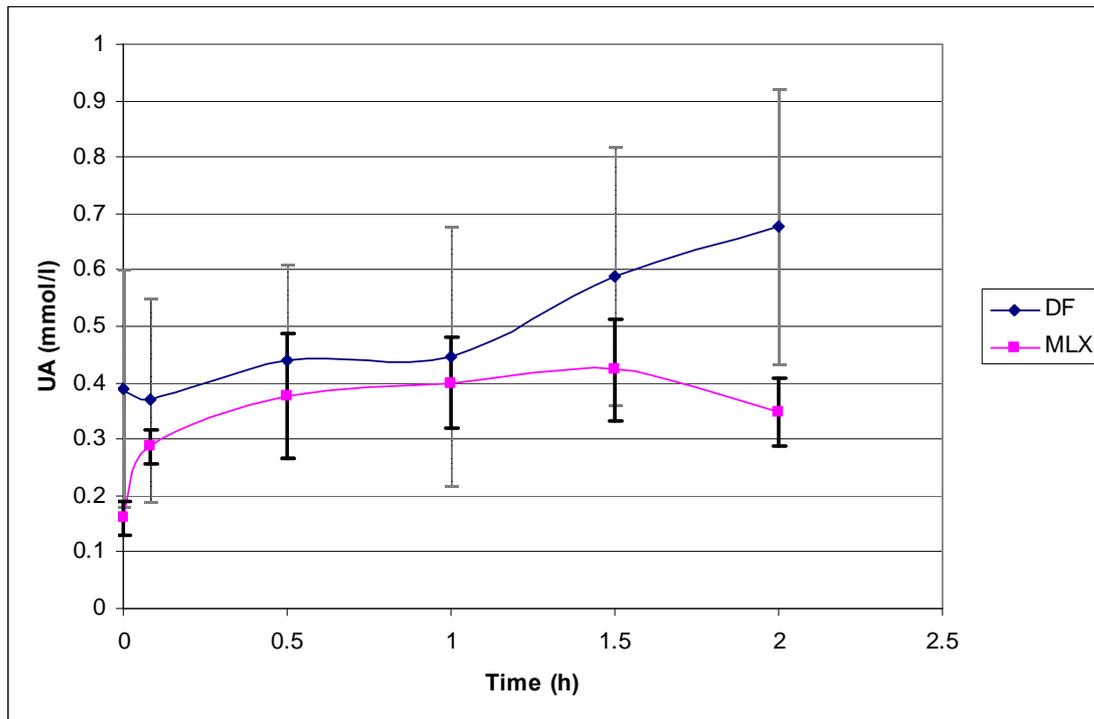


Figure 7-3: *In vivo* change in serum UA concentration for *G. corprotheres* over the first two hours following the administration of MLX (n=4) or DF (n=4)(p=0.037). Results are presented as mean \pm SEM

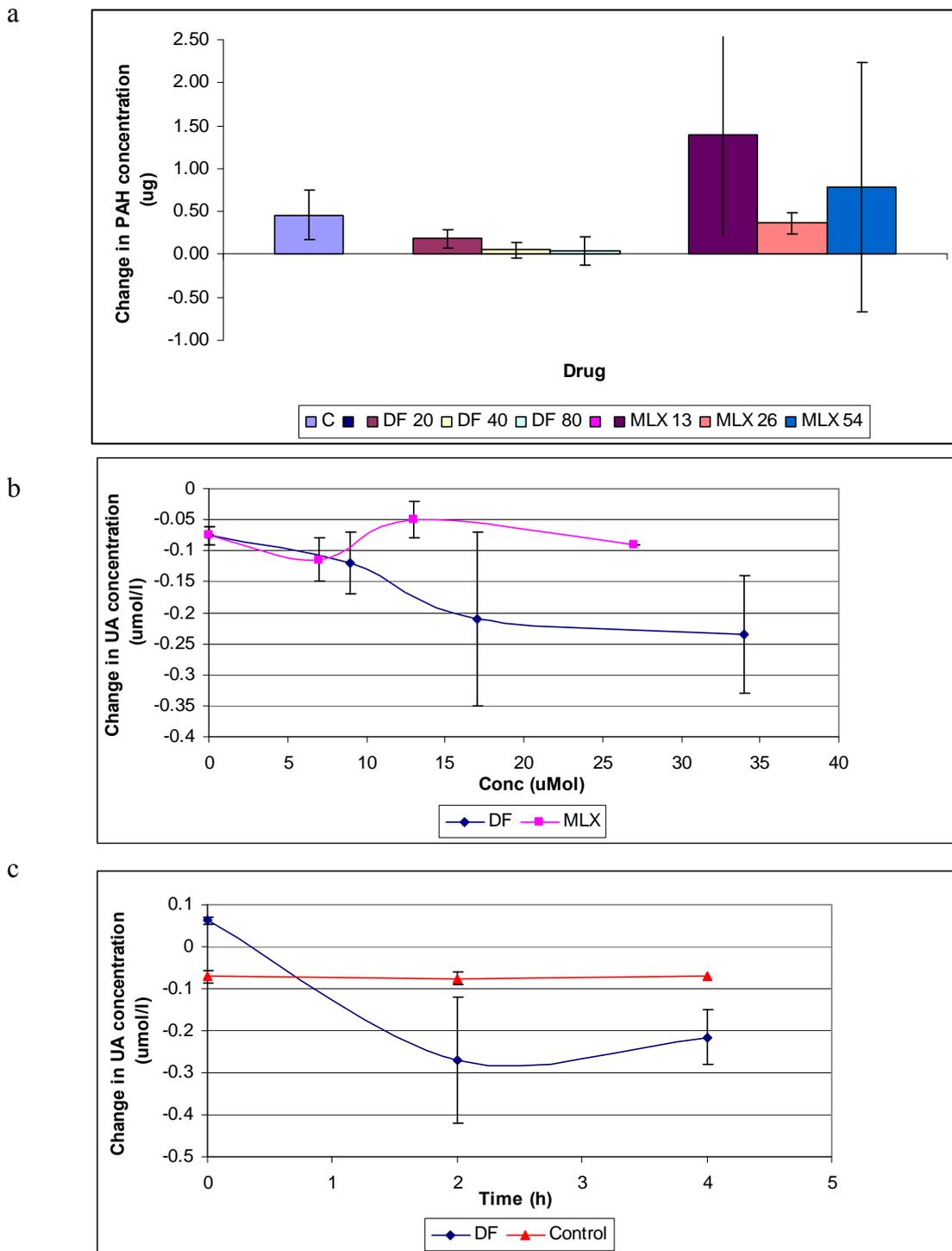


Figure 7-4: Influence of DF or MLX on UA or PAH clearance in cell cultures established in double chambered well. Results are presented as mean \pm SEM.

a) change in PAH concentration for chicken RTE cells following 2 hours of incubation with DF or MLX; b) change in UA concentrations for vulture RTE cells following incubation with DF and MLX for two hours c) Change in UA concentration for vulture RTE cells, 2 and 4 hours following the removal of DF from chamber.



CHAPTER 8: General Discussion

8.1 Hypothesis 1: Meloxicam as a vulture safe alternate

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

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

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



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



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

In the initial step in establishing diclofenac's mechanism of toxicity, the acute toxic effect of the drug was evaluated in the domestic fowl in the attempt of validating this easily obtainable species as a surrogate model. Following intramuscular administration at various doses, the injected fowls showed identical clinical signs, time to clinical signs, clinical pathological changes and necropsy changes together with similar histopathological lesions albeit more severe in the chicken. A major difference was, however, present in the LD₅₀ as the fowl was approximately twenty-fold less sensitive. Additionally the pharmacokinetic profiles were very different with the half-life in the fowl being substantially shorter. Unfortunately with the pharmacokinetics and LD₅₀ being markedly different, the domestic fowl could not be used as a surrogate in establishing the safety of other NSAIDs as they will not be sensitive indicators. However, since the clinical and necropsy findings were almost identical the domestic chicken was used in subsequent studies aimed at establishing the mechanism of drug toxicity.

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



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

With the chicken being a tested model for establishing the mechanism of toxicity, three theories were evaluated. In the process of evaluating the theory by Meteyer *et al.* (2005), an extensive literature review showed inconsistencies in the theory proposed as the valve functions physiologically in an opposite manner to that proposed *viz.* it is the open valve that predisposes the kidney to toxicity and not a closed valve as proposed. However with a slight modification to the theory, the site of drug toxicity could possibly lie at the level of the renal portal vein and not its valve. With the renal portal vein providing up to 75% of the blood supply to the avian kidney, it is possible that prolonged venoconstriction in these vessels could induce ischemia of adequate severity to induce necrosis of the renal proximal convoluted tubules. With the NSAIDs being known inducers of vessel contractility, through the inhibition of vasodilative prostaglandin E₂ synthesis, the level of inhibition would therefore most likely lie at the level of the COX enzyme.

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



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

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

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

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



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

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

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



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

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

8.3 Further studies

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

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