



Diclofenac toxicity in Gyps vulture is associated with decreased uric acid excretion and not renal portal vasoconstriction

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ABSTRACT

Diclofenac (DF), a non-steroidal anti-inflammatory drug (NSAID), is largely regarded as one of the most devastating environmental toxicant in recent times, after accidental exposure via their food-chain lead to massive mortalities in three vulture species on the Asian subcontinent. Although the use of diclofenac was recently banned on the Indian subcontinent, following the favourable safety profile of meloxicam, its mechanism of toxicity remains unknown. In an attempt to establish this mechanism, we test three hypotheses using models established from either the domestic chicken (*Gallus domesticus*) or the African White-backed vulture (*Gyps africanus*). We demonstrate that both DF and meloxicam are toxic to renal tubular epithelial (RTE) cells following 12 h of exposure, due to an increase in production of reactive oxygen species (ROS), which could be temporarily ameliorated by pre-incubation with uric acid (UA). When cultures were incubated with either drug for only 2 h, meloxicam showed no toxicity in contrast to diclofenac. In both cases no increase in ROS production was evident. In addition, diclofenac decreased the transport of uric acid, by interfering with the *p*-amino-hippuric acid (PAH) channel. We conclude that vulture susceptibility to diclofenac results from a combination of an increased ROS, interference with UA transport and the duration of exposure.

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1. Introduction

Three species of vultures endemic to South Asia are in grave danger of extinction across the Asian subcontinent. Populations of Oriental White-backed vulture (*Gyps bengalensis*), Long-billed vulture (*G. indicus*) and Slender-billed vulture (*G. tenuirostris*) have declined by more than 97% in India, Pakistan and Nepal, with the result that all three species have been classified by the IUCN, the World Conservation Union, as being critically endangered (Satheesan 1999; 2000; Prakash 1999; Prakash et al., 2003; Pain et al., 2003). Although the devastation was first noticed in the early 1990s, it was not until 2004 that Oaks et al. linked the vulture deaths to the veterinary use of diclofenac (DF), a non-steroidal anti-inflammatory drug (NSAID) (Shultz et al., 2004; Oaks et al., 2004). This causal relationship was subsequently confirmed under controlled experimental conditions in both captive Asian and African white-backed vultures (*G. africanus*) (Oaks et al., 2004; Swan et al., 2006b). More recently the Egyptian vulture (*Neophron percnopterus*) and red-headed vulture (*Sarcogyps calvus*) have also been shown to be declining (Cuthbert et al., 2006).

Although the vulture population decline has been conclusively linked to the veterinary use of DF, the drug mechanism for the species

crash has yet to be explained. The latter is mainly confounded by the relatively safe use of diclofenac in the medical industries for a number of years (Roberts and Morrow, 2001). More importantly the reason why the LD₅₀ of 0.098 to 0.225 mg/kg reported in vulture is substantially lower than reported in rodents is also unknown (Swan et al., 2006b). However, with the necropsies undertaken showing the common finding of diffuse visceral gout and proximal convoluted tubular damage, it has been concluded that kidneys or its supportive vascular system are the site of toxicity (Meteyer et al., 2005; Oaks et al., 2004; Swan et al., 2006b). The latter is not completely unexpected as diclofenac is known to be a nephrotoxic agent at higher doses in people (Hickey et al., 2001).

Meteyer et al. (2005) proposed the inhibition of renal prostaglandins (PG) and subsequent closure of the renal portal valves as causes of severe renal ischemia and nephrotoxicity. In this theory, toxicity was related to the unique avian blood supply, in which a large proportion of the blood supply to the renal proximal convoluted tubules arises from the hind-quarters via the renal portal system (Burrows et al., 1983). More importantly within these blood vessels, structures known as renal portal valves regulate renal perfusion by shunting blood directly to the caudal vena cava under conditions characterised by stress. Meteyer et al. (2005) suggested that a decrease in the formation of the vasodilatory prostaglandins (PG), via DF's cyclo-oxygenase inhibitory activity, could promote valve closure thereby shunting blood away from the kidney and thereby promote renal ischemia. Although the influence of the drug on PG is well described in

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literature, it has also been shown that the valve needs to open to shunt blood away from the kidney (Burrows et al., 1983). Furthermore with valve control being mediated by the beta adrenergic and muscarinic receptors (Burrows et al., 1983), and not the prostaglandins, Meteyer hypothesis appears to be inaccurate. The theory does, however, have merit in that renal vascular tone is maintained by PG making it plausible that DF could influence the PGs mediating venodilation thereby induce ischemia (Dunn and Hood, 1977).

For the second theory advanced, Ng et al. (2006) used rodent kidney epithelial to demonstrate that DF could be nephrotoxic due to oxidative damage to renal tubular cell (RTE) mitochondria with subsequent activation of the apoptotic cycle. In a second study, Ng et al. (2008) were also able to demonstrate that both DF and meloxicam (MLX) could induce cellular apoptosis, with MLX being the less toxic molecule. Although the theory explained the clinical signs evident, the study failed to consider possible differences between mammalian and avian physiology.

In addition, we propose that toxicity may be related to drug's ability to influence UA excretion as studies by Naidoo et al. (2007) demonstrated that the main clinical signs of depression, following exposure to diclofenac, was a direct result of the marked increase in plasma uric acid concentrations. Since the mammalian kidney is influenced by the uricosuric effects of DF and MLX through the inhibition of the epithelial cell's 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. The latter is plausible as similar PAH and MRP channels are involved with UA excretion in the chicken (birds do not reabsorb UA) (Khamdang et al., 2002; Rafey et al., 2003; Dudas et al., 2005; Enomoto and Endou, 2005; Mount et al., 2006). In addition both diclofenac and phenylbutazone have been shown to induce uricemia in the chicken (Berger et al., 1960; Naidoo et al., 2007).

To test the above hypotheses, toxicity assays were undertaken using tissue harvested from the domestic chicken, a previously validated model. The majority of assays used MLX as a control as this NSAID was previously established safe in Gyps vultures (Swan et al., 2006a).

2. Material and methods

2.1. Chemical and consumables

Diclofenac, Meloxicam, Penicillin, Streptomycin, Dichlorofluorescein diacetate (DCFH-DA), Di-methyl sulphoxide (DMSO), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), trypsin, norepinephrine (NE), NaCl, KCl, MgSO₄, NaHCO₃, KH₂PO₄, 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 anopore membrane culture inserts were obtained from NUNC, South Africa.

2.2. Animals

The Broiler chickens (*G. domesticus*) ($n=10$) used in the study were raised at the Poultry Reference Laboratory (Onderstepoort, South Africa) on a commercial broiler diet and water *ad lib*. The chickens were euthanized at 5 weeks of age by cervical dislocation. One African sub-adult White-back vulture (*G. africanus*) 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 intraperitoneally. Euthanasia was performed using pentobarbitone intravenously.

2.3. 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 (Naidoo et al., 2007, 2008a).

2.4. Organ bath studies

The cranial renal portal vein was dissected away from the parenchyma of the chicken kidney. The vein was suspended in physiological saline solution (PSS) containing calcium and contractile studies undertaken as previously described (Hessellund et al., 2003). The dissected vessels were attached onto the force transducer of a myograph (Danish Myo Technology) and allowed to equilibrate for 60 min in oxygenated PSS (5% CO₂ in air). Initial contraction was stimulated with potassium chloride and thereafter with NE (10 μmol). Once the basal NE contractile level was established, DF (0.42 μmol) or MLX (0.66 μmol) were evaluated in combination with NE (10 μmol). The effect of the drugs on each tissue was repeated three times at the same dose. Prior to the addition of each treatment, the wells were thoroughly flushed at least three times, allowing the tissue to equilibrate prior to re-stimulation with NE+drugs. NE was selected as the agonist as NE has been shown to have contractile response in the kidneys of birds (Jungbluth et al., 1994). In addition studies in the equivalent amphibian structure indicate the renal portal veins are predominantly NE responsive (Judith and Gibbins, 1983). DF was tested in quadruplicate and MLX in duplicate due to a failure in the tissue to contract despite being harvested and maintained under the same conditions.

2.5. In vitro RTE assay

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

2.5.1. Establishing of cell cultures

From the chickens and the vulture, the kidneys were harvested immediately after euthanasia, minced with a scalpel blade and incubated overnight in DMEM with 1 mg/ml of collagenase at 37 °C (Freshney, 1987). The following morning cell homogenates were passed through a 250 and 38 μm filter to isolate the RTE cells (Trifillis, 1999). The isolated cells were washed three times by centrifugation at 200 ×g with PBS and re-suspended in DMEM supplemented with 10% FCS, streptomycin and penicillin G (Trifillis, 1999).

2.5.2. Toxicity assay

Chicken RTE cells, 200 μl (10⁵ cells/ml) were seeded into 96-well plates (Lu et al., 2004). The assays were allowed to establish over 48 h, prior to incubation with drug. Following the predetermined intervals of 12, 24 and 48 h, the cultures were incubated with MTT (0.44 μmol) for 5 h (Lu et al., 2004; Orfila et al., 2000; Gerlier and Thomasset, 1986). The degree of formazan formation was determined by rinsing the wells with HBSS prior to the addition of 50 μl of DMSO and subsequent reading on a Varion spectrophotometer at 570 nm (1 cm path length). Cell survival was evaluated as a percentage of viable cells of treated to untreated wells on the same plate (% Cell Viability).

To ascertain if the drug toxicity was related to the duration of exposure, and therefore the pharmacokinetic profile of DF or MLX, chicken RTE cells were incubated with either DF or MLX for only 2 h (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.

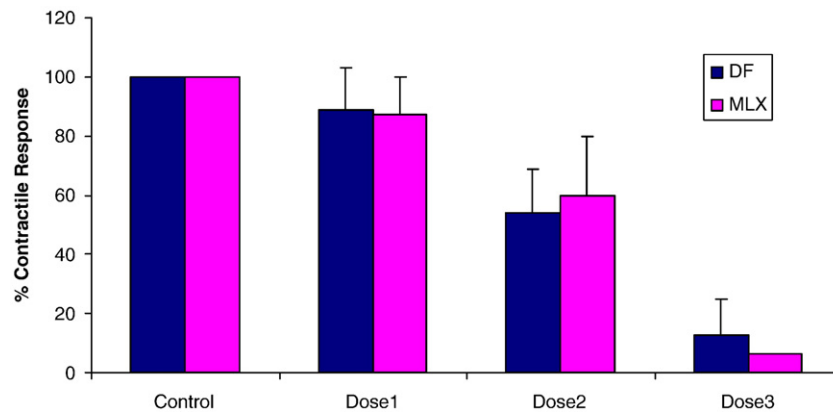


Fig. 1. Contractile response of cranial renal portal veins to norepinephrine (NE) alone (control) or the response to NE following co-incubated with either diclofenac (DF) or meloxicam (MLX) as a single dose of drug. Dose 1 to Dose 3 illustrates the additive inhibitory effect of the NSAIDs in an irreversible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

2.5.3. 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 12 h. The cell cultures were thereafter incubated with DCFH-DA, a specific indicator of intracellular ROS formation, for 30 min and washed with HBSS (Gomez-Lechon et al., 2003). The change in absorption was read at on a Varion spectrophotometer at 504 nm (Somogyi et al., 2007). 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 of treated to untreated wells on the same plate.

2.6. Influence on uric acid clearance

To ascertain if either DF or MLX had an influence on UA transport *in vivo*, 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 2 h following dosing (Naidoo et al., 2008a). Uric acid was measured with an ACE™ Uric Acid reagent kit by the rate of α -oxoglutarate conversion to L-malate (Nexet Chemistry Analyser, Alfa Wasserman, Bayer SA).

To determine the *in vitro* influence on UA transport, chicken or vulture RTE cells, 500 μ l (10^6 cells/ml) were seeded onto the anopore membrane culture inserts, in 24 well microplates and fed every three days (Ichida et al., 2003). After seven days of incubation, the culture medium was replaced with the HBSS transport medium as previously described (Ichida et al., 2003; Sutterlan and Laverty 1998; Waugh, 2006). 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 chicken RTE assays were exposed to various concentrations of DF ($n=4$) and MLX ($n=2$) following 2 h of incubation to ascertain their effect on PAH excretion. PAH was used as it is a specific transport substrate for the OAT transporter. PAH concentrations were analysed as previously described (Agarwal, 2002). The change in concentration in each well was established as the difference from the start to the stop of incubation. The net transport was calculated as the change in the basolateral concentration subtracted from the apical change.

The assay was subsequently repeated with the limited available vulture RTE cells with UA (Analysed as above). Net excretion was determined as for PAH. The bird in question was an African White-backed vulture (*G. africanus*). The kidney tissue was harvested as described above.

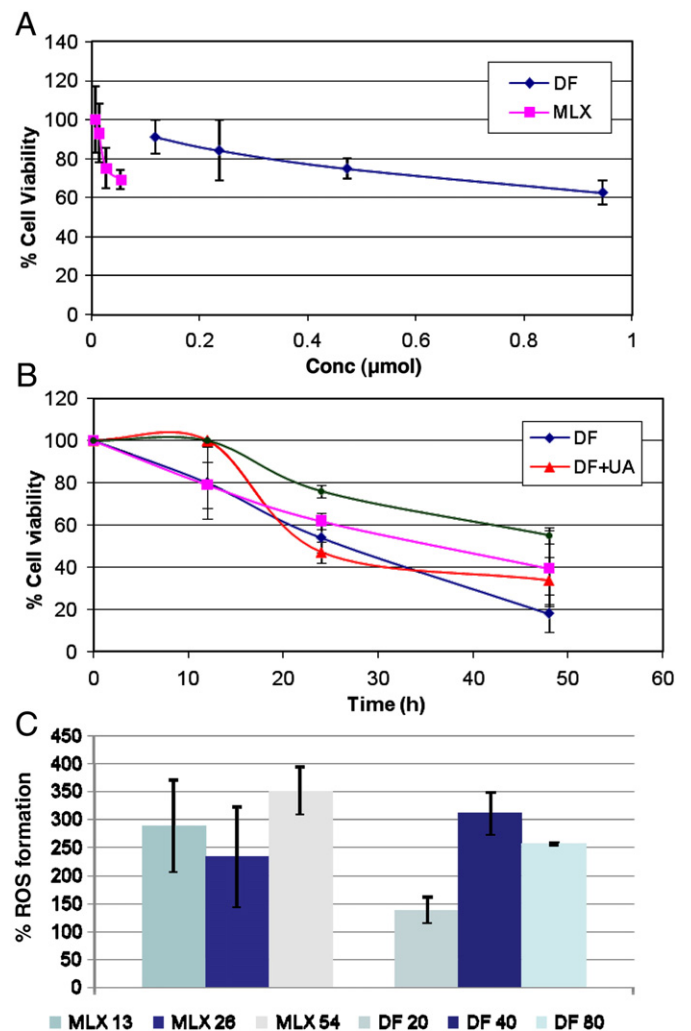


Fig. 2. Direct cell toxicity of DF and MLX following variable periods of incubation and/or concentrations using the MTT assay. Results are presented as mean±SEM. A) chicken RTE cell viability following 12 h of incubation with DF or MLX at four concentrations; B) chicken RTE cell viability following incubation with DF (470 nmol) or MLX (13 nmol) in the absence and presence of UA over 48 h [+ UA-indicates that the cultures were incubated concurrently with UA for the assay]; C) ROS production measured within RTE cells using DCFH-DA after 12 h of incubation. Results are presented as a percentage of the control±SEM.

2.7. 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 ANOVA and the post-hoc Dunnett's *t*-test. For organ baths, PAH transport and *in vivo* change in UA excretion difference over time were compared to pre-drug concentration using the paired *t*-test (SPSS 13, SPSS Inc.).

3. Results

3.1. Organ bath studies

When the venous tissue was pre-incubated with either MLX ($n=2$) or DF ($n=4$), the contractile response of the tissues were significantly lower ($p=0.028$ for DF) than the response to NE in the absence of NSAID (Control) i.e. they promoted vasodilatation (Fig. 1). Additionally the reduced contractile responsive was non-reversible despite complete flushing of the wells over a period of time.

3.2. *In vitro* cell toxicity studies

The chicken RTE cells were susceptible to the toxic effects of DF and MLX in a concentration ($n=9$ /dose/drug) and time ($n=9$ /dose/drug) responsive manner (Fig. 2), with the first signs of toxicity being evident as early as 12 h post-incubation. In all cases toxicity was associated with an increased production of ROS from 6 h post-incubation to peak by as much as 200% by 12 h post-incubation (Fig. 2).

For the cultures incubated with uric acid, the first signs of toxicity were only evident at 24 h post-incubation. The ROS production was not quantifiable in these chambers as UA is known to interfere with the DCFH-DA reagent.

3.3. Uric acid clearance

The two groups of birds had different UA concentrations at zero hours. This difference was not significant ($p=0.4$) and also within the

normal range established for the White-backed vulture (Naidoo et al., 2008b). While both MLX and DF appeared to induce an increase in plasma UA ($n=4$ /drug) (Fig. 3), only the DF effect was significantly differently from the pre-dosing sample with the 2 h time point being the most significant ($p=0.037$).

All three concentrations of DF ($n=4$) significantly decreased net PAH transport, ($p=0.021$) in a non-dose responsive manner (Fig. 4). Although meloxicam did appear to influence PAH clearance, the effect was not significant ($p=0.3$). To rule out interspecies differences, the assay was repeated with the limited available vulture RTE cells using UA. All three concentrations of DF ($n=2$) tested, promoted a concentration dependent decrease in UA clearance. In contrast MLX ($n=2$) had a weak non-significant biphasic effect (Fig. 4).

4. Discussion

From the organ bath studies it is evident that neither DF nor MLX had the potential to enhance vascular contractility in the bird tissue. This finding was not considered unusual as a similar effect was evident when rat portal veins were exposed to diclofenac (Shimamura et al., 2005). Although the finding is contradictory to glomerular arterial physiology in mammals, it can be explained by finding of Peredo (2003) who demonstrated that indomethacin decreased mesenteric vasoconstriction by inhibiting contractile PGI_2 production via cyclo-oxygenase (COX) inhibition (Peredo, 2003). With both diclofenac and meloxicam being COX inhibitors it will explain the similar effect to indomethacin.

For the *in vitro* toxicity cell death was evaluated by the inability of the cell culture to reduce the dye MTT. With the mitochondria being the only organelle capable of reducing MTT, to formazan, the resultant cell death could only be due to the death of the cellular mitochondria. This is in support of the findings of Ng et al. (2006, 2008) especially with the toxicity being associated with a 200% increase in ROS production. For both diclofenac and meloxicam, the avian primary cells were much more susceptible than the mammalian cultures as reported by Ng et al. (2008) (>100 fold, based on the concentration reported to kill 40% of the cell culture), and was in line with the lower median lethal dose (LD_{50}) reported for the chicken (Naidoo et al., 2007). Unlike Ng et al. (2008), MLX was toxic to the avian cells at much lower concentrations than DF. However, since the concentrations tested were based on the true plasma concentrations from the chicken, and with both drugs inducing the same degree of cell death

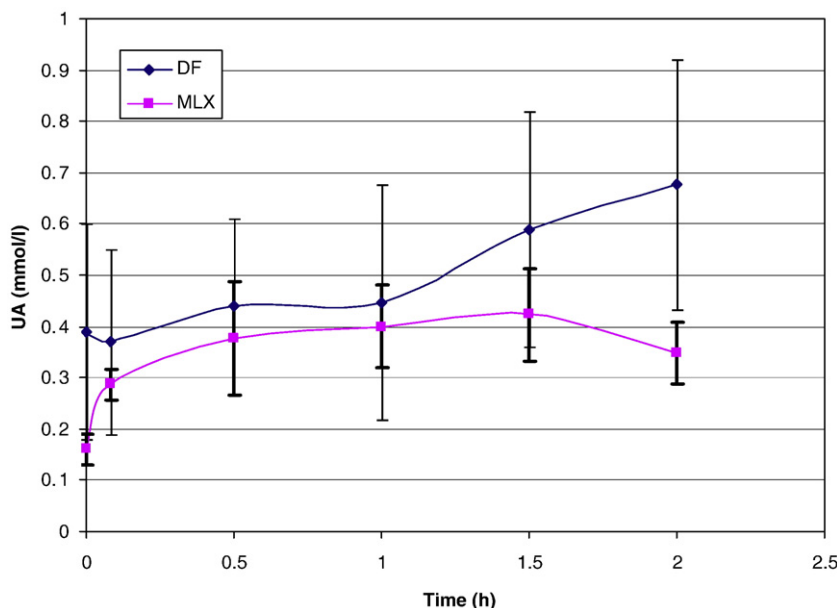


Fig. 3. *In vivo* change in serum UA concentration for *G. corprotheres* over the first 2 h following the administration of MLX ($n=4$) or DF ($n=4$). Results are presented as mean \pm SEM.

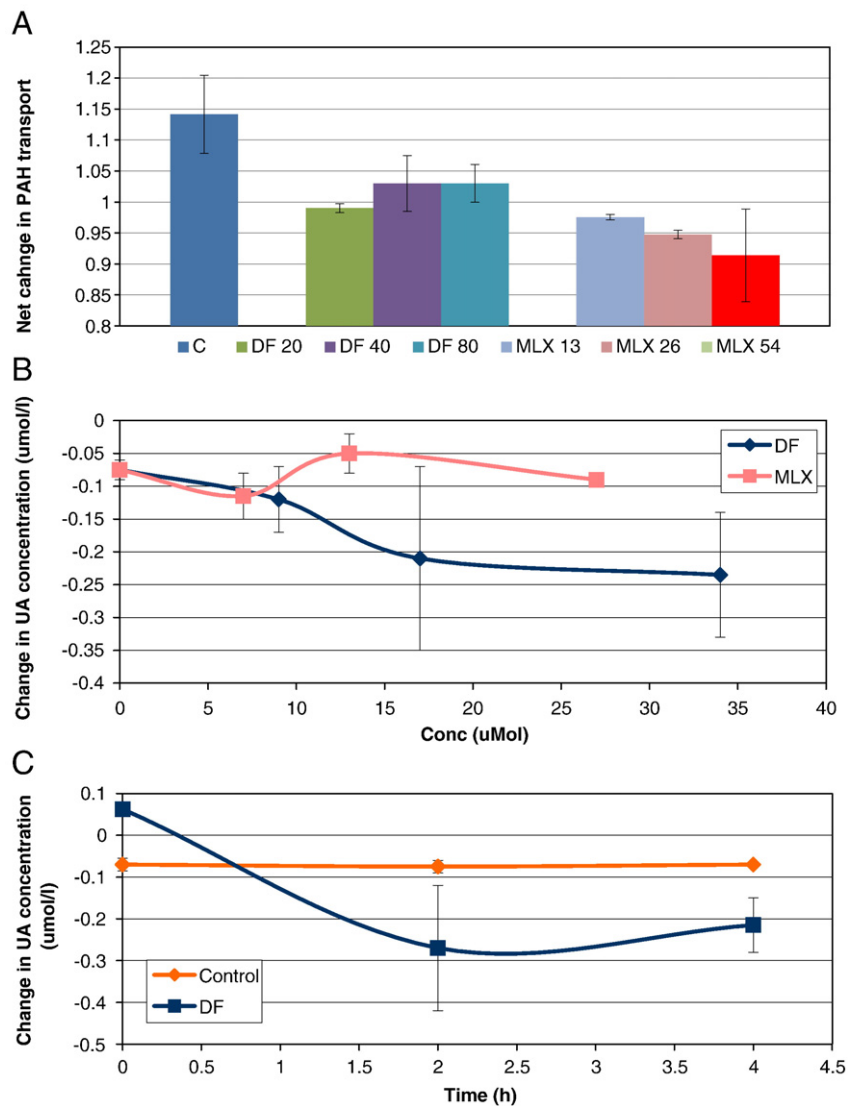


Fig. 4. Influence of DF or MLX on UA or PAH clearance in cell cultures established in double chambered well. Results are presented as mean \pm SEM. A) change in PAH concentration for chicken RTE cells following 2 h of incubation with DF and MLX; B) change in UA concentrations for vulture RTE cells following incubation with DF and MLX for 2 h; C) Change in UA concentration for vulture RTE cells, 2 and 4 h following the removal of DF from chamber.

in vitro, we conclude that MLX and DF can both be toxic to the kidneys RTE cell to the same extent.

While mitochondrial toxicity with subsequent ROS formation is a plausible mechanism, the cultures in the above assay were continuously exposed to DF and MLX for 12 h, which failed to consider the short half-life of 0.6 h of DF in the chicken i.e. chicken toxicity was not related to long-term exposure. In the case of MLX no vulture was exposed to plasma concentrations above 5 $\mu\text{g}/\text{ml}$ for more than 0.5 h (Naidoo et al., 2007, 2008a). When cultures were exposed to DF and MLX for only 2 h, no toxicity was evident for both MLX while DF remained significantly toxic ($p < 0.05$ for all concentrations) thereby indicating an alternate or additional mechanism behind toxicity.

To ascertain if this alternate mechanism was related to the depletion of intracellular UA, as proposed, cultures were incubated concurrently with UA. In all cases cell survival was promoted for 24 h, corresponding to the first *in vivo* signs of toxicity in poisoned birds (Fig. 2) (Swan et al., 2006b). With UA being an important intracellular anti-oxidant and the culture grown in the absence of UA showing an increase in ROS, the prolonged cell survival appears to be due to the buffering offered by the exogenous UA (Bekker, 1993). The short duration of protection was, however, unexpected tending to suggest

that the UA absorption/secretion by the cell was being influenced. When the latter was studied further using the artificial renal tubules, it was evident that DF decreased the transport of UA by interfering with the PAH transporter in both chicken and vulture cells (The latter was very limited as the samples were being difficult to obtain as the species is endangered.)

The *in vitro* effect of DF was similar to the *in vivo* effects as DF stimulated an increase in plasma uric acid for the entire 2 h period. Interestingly this increase was related to the drug's half-life i.e. DF which has a long half-life ($T_{1/2} = \pm 12$ h) had a prolonged influence on plasma uric acid concentrations while the opposite was true for meloxicam ($T_{1/2} = \pm 0.5$ h).

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 h post-exposure, despite signs of toxicity seen 48 h later, indicating the possibility of irreversible channel inhibition. To ascertain if DF could inhibit transporters after drug withdrawal, the vulture RTE cells were exposed to DF for 5 h followed by complete flushing of the chambers ($n=2$), prior to commencing the transporter assay. Similar to previously results, DF induced an increase in UA

concentrations in both the apical and basolateral wells (Net decrease in UA excretion). More importantly the total concentration of UA in each well was greater than that added (Fig. 4), indicating that DF depleted the cell of UA.

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 therefore be concluded that diclofenac interferes with uric acid transport 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 pharmacokinetics half-life of 2 and 14 h in the chicken and vulture, respectively and the results from the ROS study, in which exposure to diclofenac for only periods above 12 h 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 as meloxicam, a drug known to be safe in vultures, only becomes toxic *in vitro* toxicity when exposure times is extended beyond the drugs *in vivo* mean residence time.

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