Risk of single and combined exposure of birds to non-steroidal anti-inflammatory drugs and lead

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Abstract

OBJECTIVES: Pharmaceuticals and heavy metals such as diclofenac and lead, respectively, have been identified as environmental contaminants toxic to birds and posing serious threats to declining populations of raptors worldwide. The aim of the present study was to test the hypothesis that a sublethal combination of non-steroidal anti-inflammatory drugs and lead induces more pronounced effects than single exposures in birds.

METHODS: A total of 40 Japanese quails (*Coturnix coturnix japonica*) at the age of 2 months and average weight of 180g were on a random basis divided into four experimental groups of 10 specimens (i.e., control, diclofenac, lead, and lead+diclofenac exposures). Six lead shots in the total weight of 1.5 grams were inserted into the crop on day 0 of the experiment, while a total of 5 mg/kg of diclofenac administered intramuscularly were divided into treatments on days 0 and 5. Group responses were compared using haematology and biochemistry after 10 days.

RESULTS: There was no mortality in control and both single and combined diclofenac and lead exposure groups, nor did the birds show any clinical signs of intoxication. Univariate analyses of blood parameters yielded a decrease in haematocrit in birds exposed to both substances when compared with the control, a lower haemoglobin level of the lead-exposed group, increased activity of aspartate aminotransferase in the NSAIDs-exposed group, increased activity of alkaline phosphatase in birds exposed to a combination of diclofenac and lead, and a higher phosphorus level in the lead-exposed group. The principal component analysis revealed no multivariate pattern of responses of blood parameters and did not allow separation of exposure groups from controls when the variables and samples were projected onto a two dimensional space.

CONCLUSIONS: Results of the present study can enhance understanding of combination toxicity of veterinary drugs and heavy metals in birds, i.e. a scenario that has become environmentally relevant in recent decades. Fortunately, individual blood parameter effects prevailed and no joint mortal effects were recognised in Japanese quails exposed to a combination of sublethal doses of diclofenac and lead.

ORIGINAL ARTICLE

INTRODUCTION

Diclofenac, a non-steroidal anti-inflammatory drug (NSAID) used for its analgesic, antipyretic and anti-inflammatory effects, is currently considered a significant environmental contaminant (Todd & Sordin 1988). It is extremely toxic to vultures causing visceral gout, kidney failure and death of the bird within several days of exposure (Rattner *et al.* 2008). Vultures are scavengers and their primary source of food is dead animal bodies. A direct relation of renal failure in birds ingesting residues of NSAIDs contained in cattle carrion treated with diclofenac has been shown (Oaks *et al.* 2004). Therefore, it can be responsible for declines of scavengers across Eurasia and Africa (Green *et al.* 2006; Naidoo *et al.* 2009; Oaks *et al.* 2004; Saini *et al.* 2011; Swan *et al.* 2006). Some species of vultures have recently been identified as critically endangered in association with NSAIDs (Prakash *et al.* 2007).

Lead is another important environmental contaminant and cause of intoxication in birds throughout the world (Samour 2008). Scavengers and birds of prey, many of which are of great conservation concern, are at risk of feeding on carrion of game animals killed by lead ammunition (Fisher *et al.* 2006; Hernandez & Margalida 2009; Pain *et al.* 2007). Despite its relative insolubility, digestive juices and acids allow lead absorption throughout the gastrointestinal tract (Samour 2008). This may be a long-lasting process resulting in chronic toxicity and non-specific clinical signs (Thompson 2007).

Free-ranging birds are certainly exposed to multiple stressors with synergistic, additive or independent actions (Pikula *et al.* 2010; Relyea & Hoverman 2006; Sanderson & Solomon 2009). Considering the above-mentioned reports on risk of NSAIDs and lead in wildlife, birds can most probably be affected by a combination of both. Combined effects of these pollutants have not yet been evaluated experimentally. The aim of this study, therefore, was to test the hypothesis that the adverse effects of NSAIDs and lead combine to enhance avian mortality. For this purpose we compared the effects of single and combined exposures to diclofenac and lead shots in the digestive tract of Japanese quails and evaluated the clinical signs, mortality, haematology, and biochemistry.

MATERIAL AND METHODS

Experimental design

The experiment was conducted in accordance with standard methods OECD 205 (Guideline for the testing of chemicals-Avian Dietary Toxicity Test, 1984) using the Japanese quail (*Coturnix coturnix japonica*) as a model bird species indicative of avian toxicity (Romijn *et al.* 1995). In all, 40 two-month-old Japanese quails (*Coturnix coturnix japonica*) were randomly divided into groups of ten birds for exposure to diclofenac and lead in single and combined doses after 7 days of acclimatization, i.e. control, diclofenac (NSAIDs), lead (Pb), and lead+diclofenac (Pb+NSAIDs). Exposure to lead was performed by inserting six lead shots of the total weight of 1.5 g into the crop (ingluvies) at the start of the experimental period of 10 days. Retention of lead pellets in the gastrointestinal tract was confirmed by recovering all six lead shots from the gizzard (ventriculus) of birds on necropsy at the end of experiment. As reported previously, absorption from the digestive tract amounts to approximately 25% of lead weight within 10 days of administration and is known to be sublethal (Pikula *et al.* 2010). Diclofenac (Dolmina inj sol 5×3 ml/75 mg, Zentiva, Czech Republic) was administered intramuscularly into the pectoralis major muscle in the dose of 2.5 mg/kg on days 0 and 5 of the experimental period. The total dose of 5 mg/kg of diclofenac was supposed to exert sublethal effects according to Hussain *et al.* (2009). Non-steroidal anti-inflammatory drugs are absorbed quickly both following oral and intramuscular injection, the latter mode of administration was selected to ensure application of equal doses to each experimental bird (Graham *et al.* 2005). Japanese quails were provided with standard feed mixture and water *ad libitum*. On day 10 of exposure the birds were blood sampled for comparisons of blood parameters.

Biochemistry

Blood (2ml) was collected from the right jugular vein using a 2ml heparinised syringe (B.Brown Injekt®, Germany) and a 25 gauge needle (0.5×25 mm, Terumo Europe, Belgium), and was processed as previously described (Pikula *et al.* 2010). Haematocrit (l/l) was measured using microhaematocrit heparinised centrifuge capillary tubes. Whole blood samples were analysed using an automated analyser (SPOTCHEM™ EZ SP-4430, ARKRAY, Japan) for haemoglobin (g/l), glucose (mmol/l), total cholesterol (mmol/l), total bilirubin (μmol/l), aspartate aminotransferase (μkat/l), alanine aminotransferase (μkat/l), alkaline phosphatase (μkat/l), creatinine (μmol/l), high-density lipoprotein cholesterol (mmol/l), total protein (g/l), creatine kinase (μkat/l), calcium (mmol/l), phosphorus (mmol/l), magnesium (mmol/l), triglycerides (mmol/l), uric acid (mmol/l), lactate dehydrogenase (μkat/l), and amylase (μkat/l).

Statistical analysis

Data obtained from experimental groups were compared with those of the control using following procedures: one-way analysis of variance (ANOVA), post-hoc analysis of means by the LSD test, Levene's method to test for the homogeneity of variances, log-transformation of non-homogenous parameters prior to analysis and comparison with the non-parametric Kruskal-Wallis test. Both $p<0.05$ and $p<0.01$ were the levels used for statistical significance evaluation. Multivariate analysis of haematological and biochemical parameters were

performed using principal component analysis. Data analyses were performed using Statistica for Windows® 10 (StatSoft, Inc., Tulsa, OK, USA).

RESULTS

There was no mortality in control and diclofenac and lead exposure groups (both single and combined ones), nor did the birds show any clinical signs of intoxication. As shown in Table 1 of univariate analyses, no significant differences were found in parameters such as amylase, glucose, uric acid, triglycerides, magnesium, total protein, high-density lipoprotein cholesterol, alanine aminotransferase, creatine kinase, lactate dehydrogenase and total cholesterol. There was a decrease in haematocrit in birds exposed to both substances when compared with the control (HT_C=0.56±0.05 l/l; $HT_{Pb+NSAIDs}$=0.48±0.06 l/l; p<0.03). The haemoglobin level of the lead-exposed group was significantly lower in comparison with the control group (Hb_C=147±27.12 g/l; Hb_{Pb}=112.61±19.96 g/l; p<0.01). The activity of aspartate aminotransferase significantly increased in the NSAIDs-exposed group (AST_C=2.86±1.14 μkat/l; AST_{NSAIDs}=4.58±1.89 μkat/l; p<0.003). There was an increase in activity of alkaline phosphatase in birds exposed to a combination of diclofenac and lead (ALP_C=17.31±5.53 μkat/l; $ALP_{Pb+NSAIDs}$=21.59±2.59 μkat/l; p<0.04). The phosphorus level was higher in the lead-exposed group (P_C= 1.64±0.22 mmol/l; P_{Pb}=2.33±0.83 mmol/l; p<0.008). Comparing individual experimental groups of Japanese quails against the control group, significant differences included aspartate aminotransferase and calcium in diclofenac (NSAIDs)-exposed birds, haemoglobin, creatinine, calcium and phosphorus in lead (Pb)-exposed birds, and haematocrit, bilirubin, alkaline phosphatase, and calcium in lead+diclofenac (Pb+NSAIDs)-exposed birds. The principal component analysis revealed no multivariate pattern of responses of blood parameters to allow separation of exposure groups from controls after projection of the variables and samples onto a two dimensional space.

Tab. 1. Blood parameters in control Japanese quails and birds exposed to single and combined doses of diclofenac and lead.

Parameters	Groups of quails			
	Control	NSAIDs	Pb	Pb+NSAIDs
HT (l/l)	0.56±0.05	0.50±0.08	0.51±0.04	0.48±0.06*
Hb (g/l)	147±27.12	126.125±20.11	112.61±19.96**	132.71±24.06
GLU (mmol/l)	17.50±1.66	17.28±1.51	18.74±1.88	19.02±1.67
T_CHO (mmol/l)	5.71±1.32	2.23±0.66	6.02±1.35	6.18±1.42
T_BILL (μmol/l)	7.90±2.33	6.90±1.66	6.9±1.27	6.00±1.12*
AST (μkat/l)	2.86±1.14	4.58±1.89**	3.35±0.81	3.47±0.84
ALT(μkat/l)	0.60±0.25	0.56±0.15	0.54±0.14	0.47±0.14
ALP(μkat/l)	17.31±5.53	18.1±5.82	16.21±7.21	21.59±2.59*
CRE (μmol/l)	30.8±6.27	34.9±10.51	47.3±18.97**	36.77±12.27
HDL_C (mmol/l)	1.76±0.84	1.64±0.77	1.77±0.52	1.71±0.34
T_PRO (g/l)	31.2±3.96	27.10±3.75	29.6±7.24	30.77±7.71
CPK (mmol/l)	1.195±0.934	0.865±0.091	1.027±0.249	2.353±2.564
Ca (mmol/l)	3.12±0.69	2.55±0.21**	2.75±0.44*	2.54±0.36*
P (mmol/l)	1.64±0.22	1.84±0.44	2.33±0.83**	1.76±0.55
Mg(mmol/l)	1.01±0.08	0.98±0.27	1.01±0.14	1.13±0.52
TG (mmol/l)	2.92±0.67	2.22±0.67	3.02±1.35	2.82±0.98
UA (μmol/l)	637±201.25	652.4±172.26	541.1±144.73	539±167.21
LDH (μkat/l)	3.08±1.70	3.51±1.52	4.08±1.34	2.77±0.75
AMY (μkat/l)	3.95±2.18	3.44±2.51	2.90±2.06	2.84±1.59

Values represent mean ± SD; n=10 in each group; *p<0.05, **p<0.01 when compared against control group. HT=haematocrit, Hb=haemoglobin, GLU=glucose, T_CHO=cholesterol, T_BILL=bilirubin, AST=aspartate aminotransferase, ALT=alanine aminotransferase, ALP=alkaline phosphatase, CRE=creatinine, HDL_C=HDL cholesterol, T_PRO=total protein, CPK=creatine kinase, Ca=calcium, P=phosphorus, Mg=magnesium, TG=triglycerides, UA=uric acid, LDH=lactate dehydrogenase, AMY=amylase.

DISCUSSION

This experimental study examined the combination toxicity of non-steroidal anti-inflammatory drugs and heavy metals in birds. It was shown, however, that individual blood parameter effects prevailed and no joint mortal effects were recognised in Japanese quails exposed to sub-lethal doses of diclofenac and lead. This contrasts with the expectation based on another study that reported enhancement of avian toxicity after a combination of single sub-lethal effects of microcystins, lead and Newcastle vaccination (Paskova *et al.* 2011; Pikula *et al.* 2010). Likewise, mortality increased in the grey partridge flock infected by *Mycoplasma gallisepticum* due to co-infection by another infectious agent (Vitula *et al.* 2011) and synergistic effects between anticholinesterase pesticides and the infectious agent *Francisella tularensis* can increase the susceptibility of European brown hares and may enhance the overall wildlife mortality (Bandouchova *et al.* 2011). Responses of experimental animals to combined effects of multiple stressors are frequently lacking in ecotoxicological risk assessments (Fair & Ricklefs 2002). Importantly, there are common molecular mechanisms such as oxidative stress involved in the adverse effects of many stressors including toxins (Stohs & Bagchi 1995) as well as immune response to infectious agents (Constantini and Moller 2009).

Many authors investigated the toxicity of NSAIDs in scavenger birds and adverse effects were mostly reported in vultures (Das *et al.* 2011; Green *et al.* 2006; Meteyer *et al.* 2005; Oaks *et al.* 2004; Saini *et al.* 2011). However, there are also two papers reporting on experimental toxicity of diclofenac in avian species such as *Gallus domesticus*, *Columba livia*, *Acridotheres tristis*, and *Coturnix japonica* (Hussain *et al.* 2008; Jain *et al.* 2009). It is also the case of lead reported mostly as adverse effects of single avian exposure (Fisher *et al.* 2006; Hernandez *et al.* 2009; Pain *et al.* 2007; Samour 2008). To the best of our knowledge, the present paper reports on the first experiment to study combined avian toxicity of NSAIDs and lead, both frequent and important environmental contaminants posing a great single exposure risk for birds throughout the world.

Two-month old Japanese quails were selected as a model avian species in this experiment resulting in no mortality in control and diclofenac and lead exposure groups (both single and combined ones). Birds were also without any clinical signs of intoxication during ten days of observation. Mostly univariate exposure effects such as haematological differences and an increase in phosphorus associated with lead and higher activity of aspartate aminotransferase in the NSAIDs-exposed group were demonstrated. Interestingly, uric acid levels remained within the physiological range in both NSAIDs-exposed groups, despite the fact that change in this parameter is considered characteristic for diclofenac toxicity in birds (Naidoo *et al.* 2009). Supposedly, diclofenac may affect the kidney of various bird species differentially (Meteyer *et al.* 2005). For example, the chicken (*Gallus domesticus*) is more sensitive (Jain *et al.* 2009). The severity of toxic effects of diclofenac is dose-dependent and higher dose rates were required to produce biochemical responses and visceral gout was even not observed following 20 mg of diclofenac per 1kg of body weight in the Japanese quail (Hussain *et al.* 2008). On the other hand, vultures were highly sensitive and the dose of 0.8 mg/kg produced marked effects and mortality in *Gyps fulvus* and *Gyps africanus*, while the LD_{50} amounted to 0.1–0.2 mg/kg *Gyps bengalensis* (Swan *et al.* 2006). Susceptibility of vultures to diclofenac causing renal tubular damage results from a combination of an increased production of reactive oxygen species, interference with uric acid transport and the duration of exposure (Naidoo & Swan 2009).

Decreased haemoglobin and haematocrit levels in the present study are associated with lead-exposure and reflect the extent and efficiency of oxygen-carrying capacity of blood and thus the bird´s ability for physical performance. They are often used as indicators of condition in birds (Villegas *et al.* 2002). There was a decrease in bilirubin in lead+diclofenac-exposed birds. Biliverdin, however, is the major bile pigment in birds (Coles 2007). Diclofenac is also known to produce hepatic toxicity ranging in severity from congestion of parenchyma, fatty change and necrosis (Hussain *et al.* 2008). Aspartate aminotransferase, the most useful enzymatic indicator of avian liver disease (Coles 2007), was significantly increased in birds exposed to diclofenac in the present study. Alkaline phosphatase was increased in birds exposed to a combination of diclofenac and lead. It may be associated with both liver and kidney disease (Coles 2007). Elevation of creatinine, indicating nephrotoxicity, was reported in diclofenac poisoned birds (Hussain *et al.* 2008; Jain *et al.* 2009). Compared with controls, creatinine was higher in single and combined exposures to diclofenac and lead. However, only lead-exposed birds showed a significant increase. According to Wernery *et al.* (2004), calcium levels do not change in lead-exposed falcons, while phosphorus rises. Correspondingly, this study found significantly increased phosphorus levels in the lead-exposure group. However, calcium decreased in birds exposed to both single and combined doses of diclofenac and lead.

While lead absorbed in higher doses resulting in blood lead levels ≥0.2 μg/g can be poisonous for virtually all tissues, laboratory results indicate severe liver and kidney damage and anaemia (Kramer and Redig 1997; Wernery *et al.* 2004). As dose rates of both toxins were sublethal in the present study, responses in blood parameters were not very marked. More pronounced effects can probably result when the exposure lasts over a longer period.

Studies into multiple toxicity are not a new issue (Bliss 1939) and the combined exposure of birds to sublethal doses of individual stressors is ecologically real-

istic (Pikula *et al.* 2010). Interestingly, the multivariate analysis combining all measured haematological and biochemical parameters and summarizing their variation into a few principal components was not able to clearly separate single and combined exposure groups in this experiment. Joint actions can be observed for chemicals of the same mechanism of toxicity (Chen and Lu 2002). It seems, therefore, that diclofenac and lead exert differing modes of action over the short term of sub-lethal exposure in Japanese quails.

CONCLUSIONS

Results of the present study documented haematological and biochemical responses of Japanese quails as a model bird species following sub-lethal exposure to both single and combined doses of diclofenac, a non-steroidal anti-inflammatory drug, and lead. As no authors were engaged experimentally with this co-exposure issue, the reported data can be of importance for ecotoxicology, pharmacotoxicology and avian wildlife conservation.

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Potential Conflicts of Interest: *None disclosed.*

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