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Salmeterol or doxycycline do not inhibit acute bronchospasm and airway inflammation in cats with experimentally-induced asthma

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ABSTRACT

The objective of this study was to determine if inhaled salmeterol, a long-acting β_2 -adrenergic agonist, and oral doxycycline, a tetracycline antibiotic displaying matrix metalloproteinase (MMP) inhibitory activity, reduce airway inflammation and obstruction in cats with experimentally-induced asthma. Eight *Ascaris suum* (AS)-sensitised cats were enrolled in a prospective study in which they underwent four AS-challenges at 1 month intervals. The challenged animals were given no treatment or were treated on 4 consecutive days with either: (1) oral prednisolone (1 mg/kg twice daily), (2) inhaled salmeterol (50 μ g twice daily), or (3) oral doxycycline (5 mg/kg twice daily), according to a randomised cross-over design. Inhibition of allergen-induced early (EAR) and late (LAR) asthmatic reactions were assessed by barometric whole-body plethysmography. Cytology and measurement of MMP-2 and -9 activities were carried out on bronchoalveolar lavage fluid (BALF).

Although none of the treatments prevented the EAR, prednisolone treatment inhibited the LAR. Relative to untreated cats, the eosinophil percentage and MMP-2 activity in BALF were significantly reduced following prednisolone treatment ($P < 0.05$). Short-term therapy with either salmeterol or doxycycline had no effect on the EAR or LAR or on airway inflammation. Given the chronic nature of this disease in cats, long-term therapy may be required to produce more favourable functional and clinical outcomes.

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Introduction

Feline asthma is a common cause of respiratory distress in domestic cats and the incidence has increased over the last two decades (Ranivand and Otto, 2008). The disease is considered allergic in origin, caused by a Th2-driven hypersensitivity reaction to inhaled environmental allergens (Norris Reinero et al., 2004; Prost, 2008). There are similarities with human asthma in that cats present with chronic lower airway inflammation with attendant bouts of coughing, wheezing and/or laboured breathing due to bronchoconstriction. Our experimental model of feline asthma, which is based on sensitisation of animals to *Ascaris suum* (AS), successfully mimics the early (EAR) and late (LAR) asthmatic reactions (i.e. the acute- and late-phase bronchoconstriction that occurs within 5 min and 4–8 h of allergen inhalation, respectively), airway eosinophilia, and non-specific airway hyper-responsiveness (Kirschvink et al., 2007b; Leemans et al., 2010). Despite the usefulness of this model in elucidating the pathophysiology of the disease and in

identifying novel therapies, it remains an experimental protocol, and may not necessarily reflect all the features of naturally occurring asthma.

There is convincing evidence that bronchodilators such as the β_2 -adrenergic agonist salmeterol are useful in controlling the recurrent airway obstruction mediated by aeroallergens and this drug has been used prospectively in humans to prevent asthmatic attacks (Cazzola and Matera, 2007). Although salmeterol delivered by pressurised metered-dose inhaler is effective in preventing carbachol-induced bronchospasm in healthy cats (Leemans et al., 2009), its potential benefit on the EAR and LAR remains unexplored. A salmeterol–fluticasone propionate combination induced a twofold greater decrease in bronchoalveolar lavage fluid (BALF) eosinophil percentages in asthmatic cats than corticosteroid treatment alone (Leemans et al., 2011). However, it remains unclear whether differences in the magnitude of this decrease are related to the anti-inflammatory potential of salmeterol or alternatively to the steroid-potentiating properties of this drug.

Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are key regulators of extracellular matrix (ECM) turnover, and are likely participants in the pathogenesis of asthma (Gueders et al., 2006). Single allergen challenge

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leads to significant increases in eosinophil count, and MMP-2 and -9 (also termed gelatinase A and B) activity in the BALF of cats experimentally sensitised to AS (Kirschvink et al., 2007b; Leemans et al., 2007), and more recent work has shown that oral or inhaled steroids reduce airway inflammation and hyper-responsiveness (probably through downregulation of MMP-2) in cats with experimentally-induced asthma (Leemans et al., 2011). Given that excessive ECM breakdown may be involved in the pathogenesis of feline asthma, there is a rationale for assessing therapies aimed at selectively inhibiting this process.

Besides antimicrobial activity, doxycycline can inhibit MMPs belonging to the collagenase (MMP-1, -8 and -13) and gelatinase (MMP-2 and -9) subfamilies (Uitto et al., 1994; Hanemaaijer et al., 1998; Smith et al., 1999). Doxycycline therefore has potential as an adjunct therapy for diseases involving excessive ECM breakdown such as periodontitis (Gapski et al., 2009), aortic aneurysms (Abdul-Hussien et al., 2009), colorectal cancers (Onoda et al., 2004), rheumatoid arthritis (O'Dell et al., 2006) or asthma (Gueders et al., 2008). In murine models of acute asthma, exposure to inhaled or oral doxycycline reduces airway inflammation and hyper-responsiveness by in situ modulation of MMP-derived enzymatic activity (Lee et al., 2004; Gueders et al., 2008). However, the results of these pre-clinical studies have not yet been validated in human or feline asthmatics.

This prospective study aimed to compare the effects of oral prednisolone, oral doxycycline (a tetracycline antibiotic displaying MMP inhibitory activity) and inhaled salmeterol (a long-acting β_2 -agonist with presumed anti-inflammatory properties) on: (1) allergen-elicited EAR and LAR, (2) airway inflammation, and (3) airway hyper-responsiveness to carbachol in cats with experimentally induced acute asthma. Assuming that salmeterol has a dual therapeutic action, it might be expected to have beneficial effects on

both lung function and airway inflammation. Additionally, we hypothesised that a therapeutic strategy based on MMP-inhibition would attenuate airway eosinophilia and hyper-responsiveness.

Materials and methods

Animal selection and sensitisation

Eight shorthair cats (four neutered males and females, respectively) aged 47 months and with a mean bodyweight of 4.7 ± 0.8 kg (Harlan), from a colony of cats experimentally sensitised to AS (Kirschvink et al., 2007b), were enrolled in this prospective study over a 5 month period. The selected cats were between 11 and 29 months of age at initial sensitisation (median age, 13 months), and had 8–16 AS challenges in previous studies (median, 11), and their latest AS challenge had been 4 months previously.

The selected animals exhibited no evidence of respiratory disease in the 6 weeks prior to study commencement. All cats developed an asthma-like phenotype after acute exposure to AS allergen, namely, eosinophilic airway inflammation (eosinophil% in BALF >18%) (Padrid et al., 1991; Hawkins et al., 1994) and demonstrable bronchial hyper-responsiveness to carbachol defined as a decrease in C-Penh₃₀₀ after AS relative to saline-challenge. C-Penh₃₀₀ was the chosen end-point i.e. the dose of carbachol that increased the enhanced pause (Penh), a unitless index of airflow limitation, to 300% of the post-saline value.

The study was approved by The Animal Ethical Committee of the University of Liège, Belgium (authorisation number 74). The animals were housed and cared for according to National Guidelines and as advised by the European Council for the Care of Laboratory Animals. Each cat was weighed weekly and underwent regular clinical examination including careful lung auscultation.

Study design

The eight selected cats entered a randomised controlled cross-over study that commenced with a 6 week assessment period, followed by four treatment phases separated by 4 week recovery periods. The initial assessment period was designed to validate in a preliminary efficacy trial the dose of inhaled salmeterol (50 μ g) used in subsequent experiments, and to identify cats developing an EAR to allergen inhalation (Fig. 1). Based on current recommendations in human paediatrics (Bisgaard,

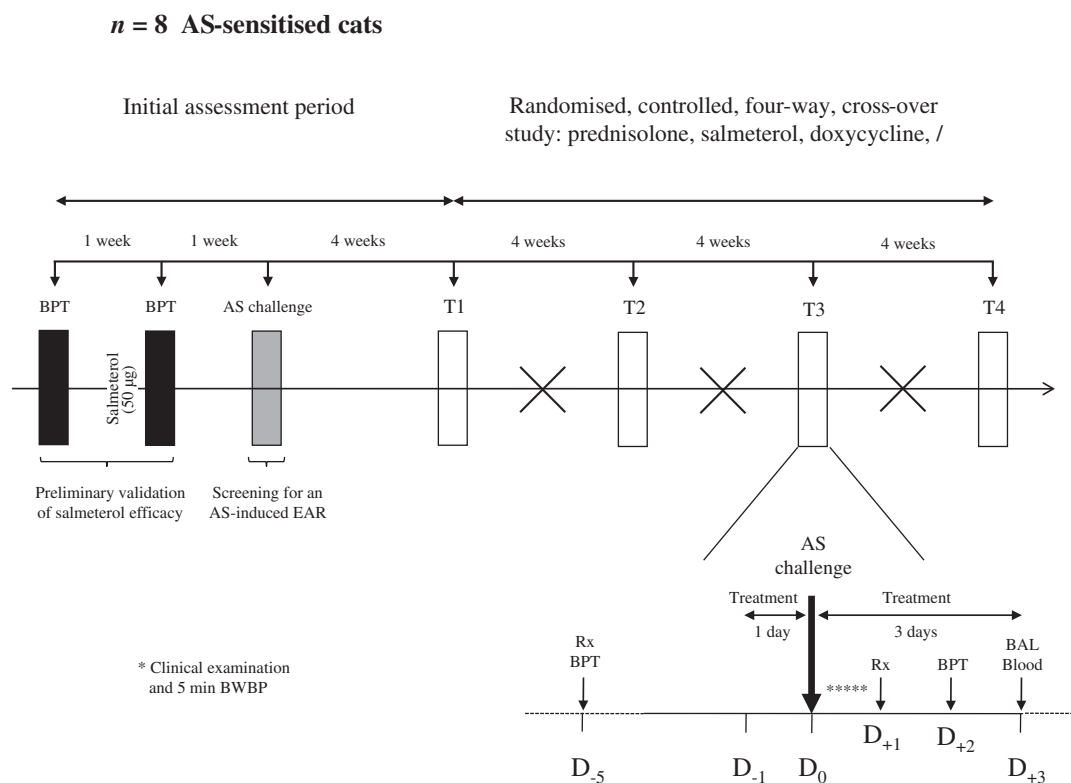


Fig. 1. Schematic overview of study design. AS, *Ascaris suum*; T1–T4, successive periods of assessment of treatment efficacy; D_x, day X prior to or following AS challenge; EAR, early asthmatic reaction; BWBP, barometric whole-body plethysmography; BPT, bronchial provocation test with carbachol; Rx, chest radiography; BAL, bronchoscopy and bronchoalveolar lavage; Blood, blood sampling.

2000), the chosen dose of salmeterol (50 µg) was tested in cats for its capacity to prevent carbachol-induced bronchoconstriction. Briefly, the eight AS-sensitised cats underwent two bronchial provocation tests (BPT) with carbachol (Sigma–Aldrich) with a 1 week interval, the first test being performed at baseline and the second 2 h after inhalation of salmeterol (Fig. 1). As reported previously (Leemans et al., 2009), airway responsiveness (AR) to inhaled carbachol was assessed by barometric whole-body plethysmography (BWBP) and was used to monitor the response to treatment.

One week later, cats were screened for an AS-induced EAR after allergen inhalation challenge. Each animal underwent successive 5 min nebulisations (Ultraneb Devilbiss 2000, Devilbiss Healthcare) with increasing AS concentrations (0.005%, 0.01%, 0.02%, 0.04% and 0.08%). Clinical examinations and 5 min of BWBP were carried out after each nebulisation which were terminated when sustained dyspnoea with Penh values >300% of baseline measurements (EAR) were recorded, or once the maximum dose (0.08%) was given.

Of the eight cats under test, five developed an EAR after allergen challenge, but three did not, despite stimulation with five doubling concentrations of allergen. Of the five responsive cats, the incremental doses of allergen leading up to the desired degree of airflow limitation were used for all subsequent challenges (cross-over trial), as this allergen exposure protocol induces highly reproducible acute alterations to pulmonary function (Leemans et al., 2010). The three unresponsive cats were arbitrarily exposed to a single 5 min challenge with 0.01% AS in subsequent challenges, as this dose of allergen is efficient in eliciting reproducible eosinophilic inflammatory responses (Kirschvink et al., 2007a,b).

Treatments commenced at the end of a 4 week recovery period and were based on a randomised, controlled four-way cross-over design. Cats were randomly assigned to receive either: (1) oral prednisolone (1 mg/kg, twice daily) (Kela Laboratoria); (2) inhaled salmeterol (50 µg, twice daily) (Serevent, GlaxoSmithKline); (3) oral doxycycline hyclate (5 mg/kg, twice daily) (Ronaxan, Merial); or (4) no treatment. On four different days, the cats underwent an AS bronchial provocation challenge. The day of allergen exposure was designated as day 0 (D₀). Each treatment was administered daily from the day before (D₋₁), until 2 days following challenge (D₀, D₊₁, D₊₂). On D₀, the treatments were given 2 h before stimulation. The cats were 'crossed-over' to the other treatments at 1 month intervals, to insure that each cat received all four treatments (Fig. 1).

Clinical examinations and 5 min of BWBP were carried out on D₀, immediately prior to AS inhalation (T₀), and at different time-points after stimulation (i.e. those resulting in the desired degree of airflow limitation), on responsive cats or after challenge with 0.01% AS in unresponsive animals. Clinical and functional responses to AS exposure were assessed at 5 min, 15 min, 1 h and 2 h after challenge, then every 2 h until 12 h post-challenge and finally at 24 h post-challenge. Chest radiography and BPT with carbachol were first performed before AS stimulation (D₋₅), and then at 24 h (D₊₁) and 48 h (D₊₂) post-challenge, respectively. On D₊₂, the airway response to inhaled carbachol was assessed 2 h after drug administration. BALF and blood were collected 72 h (D₊₃) after stimulation (Fig. 1).

Drug administration

Salmeterol was delivered to cats using a metered dose inhaler (pMDI) adapted to animal use by connecting a spacing chamber and facemask (Aerokat, Trudell Medical). The facemask was carefully applied to the nose and mouth and the spacing chamber was loaded with one puff at a time of salmeterol (25 µg). After each actuation of the pMDI, the facemask was held in place until the animal had taken 10 deep breaths. Compliance with inhalation therapy, assessed by counting the number of failed and successful drug administrations on the basis of previous delivery procedures, was approximately 94% at study termination. Given the risk of oesophageal injury in cats associated with use of the hyclate salt of doxycycline, oral administration of this compound was followed by approximately 5 mL of water given by syringe (German et al., 2005). No adverse reactions were reported.

Clinical scoring

The same investigator (JL), who was not aware of an animal's treatment allocation, conducted all clinical examinations and employed a semi-quantitative clinical scoring system ranging from 0 to 15. This score incorporated an animal's general health status, respiratory pattern and rate, auscultated lung sounds and the presence of spontaneous coughing. General status was scored as: 0, normal; 1, lethargic. Respiratory pattern was scored as: 0, normal; 1, moderate dyspnoea; 2, severe dyspnoea. Respiratory rate was scored as: 0, ≤50 cycles/min; 1, 50–80 cycles/min; 2, >80 cycles/min. The following keys were applied for scoring lung sounds on auscultation: increased breath sound (0, absent; 1, medium grade; 2, high grade); crackles (0, absent; 1, medium grade; 2, high grade); low-pitched wheezes (0, absent; 1, medium grade; 2, high grade); high-pitched wheezes (0, absent; 1, medium grade; 2, high grade). Coughing was scored as: 0, absent; 1, ≤5 successive cough cycles; 2, >5 successive cough cycles.

Barometric whole-body plethysmography and determination of airway responsiveness

Respiratory parameters were measured using BWBP on unrestrained conscious cats as previously described (Kirschvink et al., 2006). The following variables were recorded: respiratory rate (RR) in cycles/min; inspiratory (Ti) and expiratory time

(Te) in ms; relaxation time (RT) in ms (i.e. the time-point when 65% of tidal volume is expired); peak inspiratory (PIF) and expiratory pseudoflows (PEF) in mL/s; estimated tidal volume (V_T) in mL; and the estimated minute volume (MV) in mL (calculated from V_T × RR). The enhanced pause Penh was calculated using the formula:

$$\text{Penh} = ((\text{Te} - \text{RT})/\text{RT} \times \text{PEF}/\text{PIF}).$$

Airway responsiveness to inhaled carbachol was assessed using BWBP as previously described (Leemans et al., 2009). In brief, cats underwent successive challenges with incremental concentrations of carbachol (0.005–0.1%). The chosen end-point was the dose of carbachol that increased Penh to 300% of the post-saline value (C-Penh₃₀₀ %).

Thoracic radiological assessment

Ventro-dorsal and right lateral views were taken of unsedated cats as previously described (Kirschvink et al., 2007a). Each pair of radiographs was graded using a semi-quantitative scoring system (0–9), by a radiologist (FS) without knowledge of animal identity or treatment, and that reflected the severity of the bronchial (0–3), interstitial (0–3) and alveolar (0–3) patterns present.

Blood sampling, bronchoscopy and bronchoalveolar lavage

Once cats were sufficiently sedated using 0.1 mg/kg of medetomidine IM (Domitor, Orion) (Kirschvink et al., 2007b), venous blood was collected by jugular venepuncture into EDTA and sodium citrate tubes for haematology and MMP analysis, respectively. Anaesthesia was then induced and maintained using IV propofol at 1–2 mg/kg (Diprivan, Astrazeneca). BAL was performed using a paediatric 4.8 mm diameter video-endoscope (Fujinon EB-4105, ONYS S.A.), located first in the right, and then in the left, diaphragmatic lobe. Each lavage consisted of 10 mL of sterile saline at 37 °C.

Bronchoscopic findings were scored on a scale from 0 to 12 using a method adapted from Kirschvink et al. (2006). The scoring system took into account the presence of mucus in the trachea and bronchi, as well as the appearance of the mucous membranes (i.e. the existence and degree of changes such as congestion and oedema) (Table 1). The bronchoscopy scores were established by two observers (JL and FBI), without knowledge of animal identity or treatment, following video-review of each bronchoscopic examination.

Sample processing and analysis

Bacteriological examination and semi-quantitation of aerobic organisms were performed on BALF. An aliquot of BALF was mixed with gentian violet solution (50:50 [vol/vol]), and four total nucleated cell counts/sample were carried out using

Table 1
Details of bronchoscopy scoring scheme.

Features	Bronchoscopic findings
Appearance of mucous membrane	0–8
Congestion	
Trachea	0, no (or few) blood vessels visible 1, blood vessels clearly distinguishable 2, dense blood vessel network (and possibly haemorrhage)
Bronchi	0, normal 1, mild mucosal reddening 2, pronounced mucosal reddening
Oedema	
Trachea	0, no oedema 1, mild oedema with thickening of the dorsal tracheal membrane 2, pronounced oedema with thickening and protrusion of the dorsal tracheal membrane
Bronchi	0, no oedema 1, mild oedema with thickening of bronchial septa 2, pronounced oedema with thickening of bronchial septa and presence of 'blebs' on mucous membranes
Presence of mucus	0–4
Trachea (0–2) and bronchi (0–2)	0, no mucus plugs 1, few mucus plugs 2, abundant mucus plugs

a Thoma cell within 4 h of collection. Differential cell counts (%) were carried out on BALF cytopsin preparations stained with May–Grünwald–Giemsa. At least 300 non-squamous cells were counted in each sample by an observer (JL) without knowledge of animal identity or treatment. The remainder of the BALF sample was centrifuged (450 g for 10 min at 4 °C) and the supernatant stored at –80 °C until assayed for total protein concentration and MMP and TIMP activity.

BALF protein levels (mg/L) were determined using a spectrophotometric assay (Leemans et al., 2011). Gelatinolytic activity associated with MMP-2 and -9 was measured by gelatin zymography (Leemans et al., 2011). This assay detects both pro- and active forms of MMP-2 and -9. Pro-MMP-2 and -9 standards (human pro-enzyme MMP-2 and MMP-9, Oncogene), BALF and plasma samples diluted in non-reducing loading buffer were subjected to electrophoresis on 10% (w/v) polyacrylamide SDS gels co-polymerised with porcine skin gelatin (0.1% [w/v]) (Sigma–Aldrich) as substrate. The gels were washed in Triton-X-100 2% (w/v), rinsed briefly and incubated at 37 °C for 20 h in a reaction buffer containing 50 mmol/L Tris–HCl (pH 7.5) and 10 mmol/L calcium chloride. Following staining with Coomassie blue G250, the gelatin-degrading enzymes were identified as unstained zones against a blue background. Lysis bands were scanned and converted to numeric images before quantification with Scion imaging analysis software (Version 4.0.3.2, Scion). Results were expressed as arbitrary units (AU) corresponding to pixel density $\times$ mm² for the bands of proteolysis normalised by the same value calculated for internal standards.

Biological samples were assayed for TIMP-1 and -2 activity using reverse zymography (Leemans et al., 2011). TIMP-1 and -2 standards (recombinant human TIMP-1 and TIMP-2, Oncogene), BALF and plasma samples diluted in non-reducing loading buffer were migrated in 15% (w/v) polyacrylamide SDS gels co-polymerised with porcine skin gelatin (0.05% [w/v]) and conditioned medium of transfected CHO cells G 100.11 overexpressing gelatinase A. The gels were washed in Triton-X-100 and incubated in the reaction buffer as described in standard zymography procedures. After staining with Coomassie blue G250, inhibitors of gelatin-degrading enzymes were identified as blue zones against a clearer background. Scanning densitometry was performed on zymograms and the results were expressed as AU corresponding to pixel density $\times$ mm² for the lysis-resistant bands normalised by the same value as that calculated for internal standards.

Data handling and statistical analysis

Data from cat 2 during treatment period 4 (Table 2) were excluded from our analysis because of this animal's health problem that were unrelated to the experimental procedure. Thus analysis was based on either seven (prednisolone treated) or eight (untreated, salmeterol treated, doxycycline treated) cats.

Clinical and plethysmographic variables were transformed prior to subsequent calculations and statistical analyses, and were expressed in terms of their difference from baseline values (T0). The overall effect of the treatments on these parameters was evaluated by the 'area under the time-response curve' between T0 and 120 min following challenge (AUC_{0–120}), which was computed by trapezoidal integration. The protective effects of these medications were also assessed by calculating the maximal changes in clinical and functional variables within 120 min of AS stimulation. Penh and RR were considered the primary plethysmographic measures.

A statistical analysis package (SAS 9.1, SAS Institute) was used and the level of significance was set at $P < 0.05$. Data were tested for normal distribution using the Shapiro–Wilk statistic (PROC UNIVARIATE) and for homogeneity of variances using the F_{max} test. The random animal effect and fixed effects (i.e. influence of sex, treatment and the interaction of these), were investigated with a generalised linear model (PROC GLM). Post-hoc pairwise comparisons were performed using a least square mean difference test. Data not normally distributed were rank-transformed

Table 2

Occurrence of early and late asthmatic reactions in *Ascaris suum* (AS) sensitised and challenged cats that were untreated or after treatment with: oral prednisolone (1 mg/kg twice daily); inhaled salmeterol (50 µg twice daily); or oral doxycycline (5 mg/kg twice daily). EAR, early asthmatic reaction; LAR, late asthmatic reaction; Total, number of cats in each study group experiencing early and late reactions. Shaded cells, data from cat 2 which was excluded because of concurrent clinical abnormalities.

Cat	Untreated		Prednisolone		Salmeterol		Doxycycline	
	EAR	LAR	EAR	LAR	EAR	LAR	EAR	LAR
1		✓			✓		✓	
2								
3	✓		✓		✓		✓	
4	✓		✓		✓		✓	
5		✓			✓		✓	
6	✓		✓		✓		✓	
7	✓	✓			✓	✓	✓	✓
8	✓		✓		✓		✓	
Total	5/8	4/8	4/7	0/7	5/8	4/8	5/8	4/8

using PROC RANK and then analysed by the same model as for non-transformed data. Results were expressed as arithmetic means $\pm$ SEM or as medians, depending on their distribution.

Results

Preliminary validation of efficacy of inhaled salmeterol

Preventive inhalation of salmeterol (50 µg) led to significant increases in C-Penh₃₀₀ values ($n = 8$; $P < 0.01$). The median (range) C-Penh₃₀₀ rose from 0.018 (0.011–0.060)% at baseline to 0.046 (0.023–0.075)% after salmeterol administration. Changes in C-Penh₃₀₀ were of the same magnitude as those reported previously (Leemans et al., 2009).

Clinical and functional responses

At study termination, all five cats with detectable EAR at initial screening developed acute-phase allergen-induced bronchoconstriction when crossed-over to untreated conditions (Table 2). Also in line with initial screening, the three unresponsive cats (1, 2 and 5) remained unresponsive and were thus excluded from data

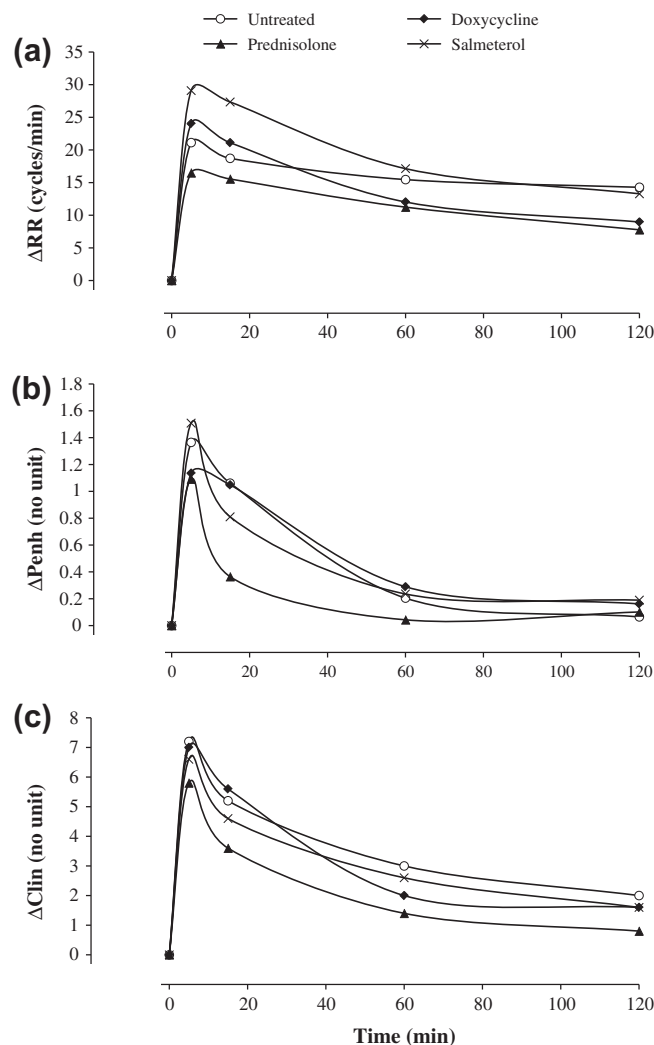


Fig. 2. Graphical illustration of differences from baseline values (Δ) of: (a) respiratory rate (RR); (b) Penh; and (c) clinical score (Clin) with time in *Ascaris suum*-sensitised/challenged cats that were either untreated or were given oral prednisolone (1 mg/kg twice daily), inhaled salmeterol (50 µg twice daily) or oral doxycycline (5 mg/kg twice daily) ($n = 5$).

analysis. Time-related changes in Δ RR, Δ Penh and Δ Clin during the EAR are illustrated in Fig. 2a–c. No significant differences were found between the four study groups, with reference to the AUC_{0–120} and maximal RR, Penh and clinical scores ($n = 5$; data not shown).

When untreated, the three unresponsive cats experienced one isolated AS-induced LAR and one responder exhibited signs of a dual asthmatic reaction. The LAR occurred as early as 4 h or as late as 12 h after exposure to allergen as evidenced by a substantial increase in Penh (>1.5) and accompanying tachypnoea of 60–70 cycles/min, moderate dyspnoea, increased breath sounds and low-pitched wheezes. Due to the small number of cats developing a LAR ($n = 4$) and the marked inter-subject variability in onset and duration of clinical and functional changes, meaningful statistical analysis could not be performed on these data and results were reported in a descriptive manner. None of the tested treatments, apart from oral prednisolone, prevented a LAR following challenge (Table 2).

Non-specific airway responsiveness

Changes in AR expressed as the difference between C-Penh₃₀₀ calculated after AS challenge (D_{+2}) and its respective baseline value (D_{-5}) (Δ C-Penh₃₀₀ = [C-Penh₃₀₀ post-challenge] – [C-Penh₃₀₀ baseline], expressed as a %), was used to compare treatments. Values of Δ C-Penh₃₀₀ in six untreated AS-stimulated cats were negative, suggesting airway hyper-responsiveness. Median Δ C-Penh₃₀₀ was significantly increased after treatment with inhaled salmeterol when compared to other groups ($P < 0.01$). Oral prednisolone attenuated airway hyper-responsiveness as indicated by an increase in median Δ C-Penh₃₀₀, but these changes were not statistically significant ($P > 0.05$) (Fig. 3).

Bronchoalveolar lavage fluid analysis

Treatments did not result in significant differences in BALF total cell counts. In untreated AS-challenged cats, the mean BALF eosinophil% reached a value close to 40% (Fig. 4), suggestive of eosinophilic airway inflammation. Compared to the respective untreated conditions (40.1 ± 7.6), the mean BALF eosinophil% was

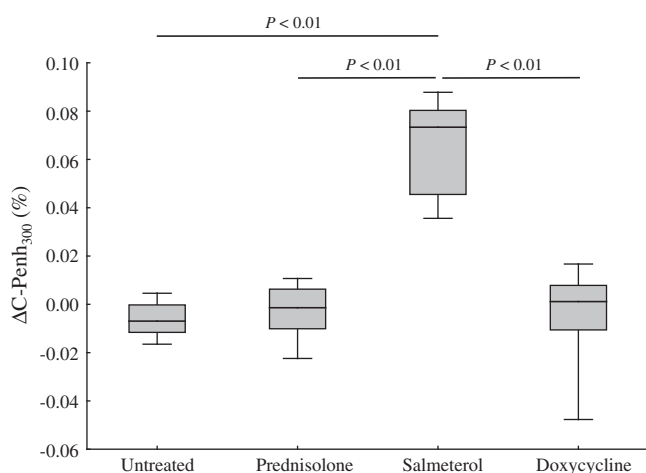


Fig. 3. Box and whisker plots illustrating changes in airway responsiveness (Δ C-Penh₃₀₀ = [C-Penh₃₀₀ post-challenge] – [C-Penh₃₀₀ baseline]) in 7–8 *Ascaris suum*-sensitised/challenged cats that were either untreated or following administration of oral prednisolone (1 mg/kg twice daily), inhaled salmeterol (50 μ g twice daily) or oral doxycycline (5 mg/kg twice daily). Central line of the box represents the median, upper and lower limits of the box represent the 75th and 25th percentiles, respectively, and the whiskers represent the maximum and minimum values. The level of significance was set at $P < 0.05$.

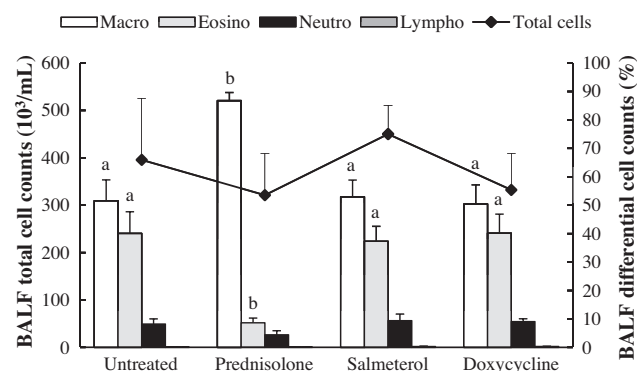


Fig. 4. Total (line graph) and differential (bar charts) cell counts in the bronchoalveolar lavage fluid (BALF) of 7–8 *Ascaris suum*-sensitised/challenged cats that were either untreated or following administration of oral prednisolone (1 mg/kg twice daily), inhaled salmeterol (50 μ g twice daily) or oral doxycycline (5 mg/kg twice daily). Data shown are means with SEM. ^{a,b}Data with different superscripts are significantly different from each other ($P < 0.05$). Macro, macrophage; Eosino, eosinophil; Neutro, neutrophil; Lympho, lymphocyte.

significantly reduced after treatment with oral prednisolone (8.6 ± 1.7 ; $P < 0.01$), while there was no such effect with either inhaled salmeterol (37.3 ± 5.2 ; $P > 0.05$) or oral doxycycline (40.2 ± 6.7 ; $P > 0.05$). The mean total protein content in BALF (mg/L) when cats were treated with oral prednisolone (120.8 ± 17.6) was significantly lower, compared with the values for the control (258.7 ± 37.7 ; $P = 0.01$), salmeterol (302.5 ± 49.1 ; $P < 0.01$) and doxycycline (237.5 ± 31.5 ; $P = 0.03$) groups.

BALF zymograms revealed gelatinolytic activity associated with pro-MMP-2 (72 kD) and pro-MMP-9 (92 kD), the latter being more prominent. No zone of proteolysis corresponding to active forms of MMP was observed. TIMP-1 was inconsistently found, and at non-quantifiable levels in both BALF and citrated plasma. Pro-MMP-2 activity in the BALF of steroid-treated cats was significantly lower than that in other groups. There were no overall treatment-related effects on BALF pro-MMP-9 and TIMP-2 activity (Table 3).

Blood analysis

Except for lymphocytes, the results of complete blood counts were within normal reference ranges. Peripheral blood lymphocytosis was observed in some steroid-treated cats (4/7), leading to a median absolute lymphocyte count ($8.6 [5.1–11.7] \times 10^9$ cells/L) slightly exceeding the upper limit of the reference range (ULg veterinary laboratory, $1.5–7.0 \times 10^9$ cells/L). None of these abnormalities were considered clinically significant. Under the conditions described, active MMP-9 was rarely present, and active MMP-2 was not detected in any plasma samples. The results indicated significantly lower plasma pro-MMP-2 activity in steroid-treated cats than in the other groups. No treatment significantly affected pro-MMP-9 and TIMP-2 activity in the citrated plasma (Table 3). No statistically significant correlations (PROC CORR with SPEARMAN option) were found between BALF and blood enzymatic activity (pro-MMP-2, pro-MMP-9, or TIMP-2) when performed separately within each treatment group.

Semi-quantitative data

Changes in total radiographic score obtained as the difference after (D_{+1}) and before AS-challenge (D_{-5}) did not differ between the four study groups (data not shown). Total bronchoscopy scores (median [range]) for untreated and treated groups were: untreated, 2.5 (1.5–4.0); prednisolone, 1.5 (0.5–2.0); salmeterol, 2.0 (1.0–3.5); and doxycycline, 3.5 (1.0–5.0), respectively. The median

Table 3

Pro- and anti-gelatinolytic activities in bronchoalveolar lavage fluid (BALF) and plasma of *Ascaris suum* (AS)-sensitised/challenged cats that were either untreated, or were treated with oral prednisolone (1 mg/kg twice daily), inhaled salmeterol (50 µg twice daily) or oral doxycycline (5 mg/kg twice daily), respectively. Data are presented as medians with ranges. Pro-matrix metalloproteinase (MMP)-2 and -9, proform of MMP-2 and -9; tissue inhibitors of metalloproteinases (TIMP)-1 and -2, type 1 and 2 tissue inhibitors of matrix metalloproteinases; AU, arbitrary unit; ND, not detected; NS, not-significant.

Variable	Untreated (n = 8)	Prednisolone (n = 7)	Salmeterol (n = 8)	Doxycycline (n = 8)	P
BALF					
Pro-MMP-2 (AU)	0.079 (0.044–0.157) ^a	0.022 (0.012–0.054) ^b	0.120 (0.051–0.157) ^a	0.057 (0.039–0.162) ^a	<0.01
Pro-MMP-9 (AU)	0.337 (0.073–1.389)	0.540 (0.220–1.400)	0.522 (0.195–2.103)	0.596 (0.205–2.267)	NS
TIMP-1 (AU)	ND	ND	ND	ND	–
TIMP-2 (AU)	0.323 (0.000–0.399)	0.211 (0.000–0.365)	0.299 (0.000–0.384)	0.216 (0.000–0.436)	NS
Plasma					
Pro-MMP-2 (AU)	0.758 (0.714–0.839) ^a	0.627 (0.383–0.696) ^b	0.783 (0.639–0.836) ^a	0.774 (0.667–0.864) ^a	<0.01
Pro-MMP-9 (AU)	0.677 (0.513–0.941)	0.728 (0.432–1.087)	0.677 (0.466–1.483)	0.708 (0.434–1.459)	NS
TIMP-1 (AU)	ND	ND	ND	ND	–
TIMP-2 (AU)	0.658 (0.422–1.736)	0.844 (0.490–1.717)	0.786 (0.453–1.953)	0.816 (0.405–1.753)	NS

^{a,b} Data with different superscripts are significantly different ($P < 0.05$).

bronchoscopy score was significantly lower in prednisolone-treated cats than in the control ($P = 0.02$) and doxycycline ($P < 0.01$) groups, respectively. Bacteriological examination of BALF found bacteria in four cases: coagulase-negative *Staphylococcal* spp. (2/31), *Staphylococcus intermedius* (1/31) or *Chryseobacterium indologenes* (1/31). These isolates were commensals, contaminants or opportunistic pathogens of low virulence and were not considered clinically significant.

Discussion

To the authors' knowledge, this is the first study to assess the effects of inhaled salmeterol and oral doxycycline on functional, inflammatory and biochemical markers in cats with experimentally-induced acute asthma. Given the dual anti-spasmodic/anti-inflammatory effects of salmeterol, we hypothesised that salmeterol inhalation might decrease the severity of allergen-induced bronchospasm and airway inflammation in asthmatic cats. However, this treatment did not prevent bronchospasm in AS-sensitised/challenged cats, nor did it demonstrate intrinsic anti-inflammatory activity. Because MMPs are thought to mediate cellular recruitment and induction of disease, we postulated that blockade of their activity by doxycycline, might be beneficial therapeutically. Again however, under our experimental conditions, neither MMP activity nor airway inflammation was significantly affected by oral doxycycline.

It should be stated that these negative findings may stem from two weaknesses in study methodology. Firstly, our model may not accurately reflect the naturally-occurring disease, where episodes of acute inflammation are super-imposed on chronically inflamed airways. Chronic exposure to AS or other clinically relevant aeroallergens (e.g. Bermuda grass allergen) may be more appropriate in assessing treatment efficacy. Secondly, the study was 'under-powered' for some end-points, notably clinical and functional variables, due to the fact that only five high-responding cats were available for statistical analysis. The power of the GLM analyses with alpha set at 0.05 was below the desired value of 0.8, indicating that the test was less likely to detect significant differences between the groups. Certainly, the risk of type two error should be taken into account when interpreting these results and drawing conclusions of potential clinical relevance.

The use of Penh as a surrogate for conventional measurements (i.e. respiratory mechanics) of airway resistance is not unanimously recognised. There are concerns about the physiological relevance of (and how to interpret quantitative changes in) this parameter (Mitzner and Tankersley, 1998; Bates et al., 2004). In the present study, Penh highly correlated with clinical score (data not shown), which is largely determined by criteria suggestive of

bronchoconstriction (coughing, wheezing and dyspnoea). The validity of Penh as a sensitive estimator of airflow limitation may not account for the lack of positive effects of the tested drugs on lung function.

Inhaled salmeterol did not lessen the severity of allergen-induced EAR and LAR in our experiment, and our results contradict current, empirical-based recommendations that support the use of inhaled bronchodilators in asthmatic cats. The fact that salmeterol, at doses similar to those used in the current study, is effective in preventing carbachol-induced and allergen-induced bronchospasm in cats and humans, respectively (Pizzichini et al., 1996; Dente et al., 1999), minimises the likelihood that an inappropriate dose was used. Impaired deposition of salmeterol within the airways may not be relevant given the preventive inhalation of the medication and, more importantly, its well-demonstrated inhibition of muscarinic-induced bronchoconstriction.

According to Schulman et al. (2004), 10–15 breaths are required to deliver uniformly a radiolabelled agent administered via a spacer and facemask into the lungs of conscious, healthy cats. On this basis, cats in our study had to take ten deep breaths after each actuation of the pMDI. Given that cats are frequently uncooperative in such settings, excessive handling may increase the risk of administration failure and bias estimates of treatment efficacy. However, this assumption seems highly unlikely since compliance with inhalation therapy in our study reached values close to 90% before allergenic stimulation.

In human asthmatics salmeterol loses its protective effects on allergen-induced EAR after 1 week of regular treatment, although the effect is partially restored when inhaled corticosteroids are co-administered (Giannini et al., 1999). Nevertheless, the lack of a significant response to inhaled salmeterol in our AS-sensitised/challenged cats might not be due to the induction of tolerance, since the duration of salmeterol intake was relatively brief and because concurrent inhalation of fluticasone did not impact on the occurrence and magnitude of the allergen-induced bronchoconstriction (Leemans et al., 2011).

The fact that the cats in the present study exhibited persistent bronchial obstruction despite prophylactic bronchodilator treatment may reflect the inability of salmeterol to reverse all the features of airway narrowing, and in particular mucus plugging of airway lumens. Sub-mucosal glands and goblet cells remain numerous in the distal aspects of the bronchial tree in the cat (Jeffery, 1978). Mucus secretion from these glands and cells is predominantly mediated by stimulation of cholinergic M₃ muscarinic receptors (Mullol and Baraniuk, 1999). Tracheal and bronchial smooth muscle from AS-sensitised/challenged cats has an increased contractile response to exogenous and endogenous acetylcholine (Mitchell et al., 1997), suggesting enhanced cholinergic

signalling and presumably associated mucus hypersecretion. Even if this remains speculative, further research is required to explore the therapeutic potential of inhaled bronchodilators in asthmatic cats.

Salmeterol and fluticasone propionate in combination are more effective than corticosteroid therapy alone in reducing airway inflammation in cats with experimentally-induced acute asthma (Leemans et al., 2011). Given the results of the current study, this therapeutic superiority might not be a direct effect of the salmeterol per se, on airway inflammation. Taken together, these findings suggest inhaled salmeterol should not be recommended as a 'monotherapy' for asthmatic cats. This recommendation is consistent with current guidelines for the treatment of asthma in humans where the use of long-acting β_2 -adrenergic agonists in isolation is not advised because of poor attendant clinical outcomes (Rider and Craig, 2006). It is possible that salmeterol functions as a steroid-potentiating agent in cats and in this respect should be viewed as a valuable adjunct therapy to inhaled corticosteroids. Further studies are required to prospectively evaluate if such combined therapy is effective in AS-sensitised/chronically challenged cats.

Inhaled salmeterol completely suppressed the allergen-induced increase in AR to carbachol in our AS-sensitised cats, with post-challenge exceeding baseline C-Penh₃₀₀ values. Most probably, this hyporesponsiveness to aerosolised carbachol results from the functional antagonism of salmeterol to muscarinic induced-bronchoconstriction given its inability to affect (by itself) airway eosinophilia or other BALF inflammatory biomarkers. Compared with the initial efficacy test, this downward shift in AR was surprisingly threefold higher despite the negative effect of allergen exposure. The reason for the improved effect may be the repeated dose scheme used in our study and the subsequent bioaccumulation of this highly lipophilic compound in the lipid bilayer of airway smooth muscle cells.

In our AS-sensitised cats, many (if not all) of the following features of the asthmatic phenotype are reproduced following acute exposure to allergen: early- and late-phase bronchoconstriction; airway hyper-responsiveness to carbachol; and airway inflammation. Oral prednisolone had no effect on the allergen-elicited EAR but completely prevented the LAR and airway eosinophilia, and caused a slight, but not significant, reduction in airway hyper-responsiveness. These results are in line with those seen in human asthmatics, in which anti-inflammatory therapy, especially inhaled corticosteroids, were found to improve all three facets of the disease (Cockcroft and Murdock, 1987; Bentley et al., 1996; Meijer et al., 1999). Our findings, together with insights from human medicine, provide a convincing link between the LAR and airway hyper-responsiveness and airway eosinophil recruitment. Nevertheless, eosinophils might not be sufficient or necessary, and non-cellular components of airway inflammation such as oedema and mucus, may be relevant in this context (Cockcroft and Davis, 2006). Neutralising interleukin-5 with a monoclonal antibody in patients with allergic asthma led to significant decreases in blood and sputum eosinophil numbers post-challenge, while there was no influence on post-allergen LAR and airway hyper-responsiveness (Leckie et al., 2000). In our model, the molecular and cellular mechanisms by which corticosteroids exert their beneficial effects on both components of asthmatic response remain to be elucidated.

Exogenous corticosteroid therapy results in transient lymphocytopenia, as a result of redistribution of circulating lymphocytes to the bone marrow (Lowe et al., 2008). In the present study, an unexpected observation was that 4/7 steroid-treated cats had mild peripheral blood lymphocytosis. On the basis of clinical history and examination, all seven cats were free of disease prior to corticosteroid administration. Moreover, the effects of prednisolone on

cellular and soluble markers of inflammation (e.g. eosinophils and MMPs) were comparable to those previously reported (Leemans et al., 2011), indicating that lymphocytosis may be simply an epiphenomenon in our steroid-treated cats.

The failure of short-term treatment with antimicrobial doses of oral doxycycline to alter MMP processes and the subsequent development of asthma in the current study contrasts with the findings from murine models. Treating mice with oral or inhaled doxycycline effectively blocks acute airway inflammation and hyper-responsiveness through in situ modulation of MMP activity, as well as chronic bronchial re-modelling (Lee et al., 2004; Gueders et al., 2008). It is unclear whether these conflicting results are due to species variations, or reflect differences in the experimental model or treatment protocol. Further investigations using larger doses, longer treatments or combination therapies in asthmatic cats will be required to fully determine the potential usefulness or otherwise of doxycycline.

Conclusions

Using a well established experimental model of feline asthma, we found that short-term salmeterol or doxycycline monotherapy had no beneficial effects on the EAR or LAR or on airway eosinophilia. Given the chronic nature of this disease in cats, more long-term therapy may be required to elicit favourable functional and clinical outcomes.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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