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Short Communication

Prophylactic effects of omega-3 polyunsaturated fatty acids and luteolin on airway hyperresponsiveness and inflammation in cats with experimentally-induced asthma

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ABSTRACT

The aim of this study was to assess the preventive effects of ω -3 polyunsaturated fatty acids (ω 3 PUFA) and luteolin supplementation on allergen-induced inflammation in eight *Ascaris suum* (AS)-sensitised cats. Airway responsiveness (AR) tests were performed and venous blood and bronchoalveolar lavage fluid (BALF) collected before and following a single (AS-stimulated) allergen exposure, as well as at the end of a 4-week treatment period, which was followed by a second AS-challenge. The ω 6/ ω 3 fatty acid ratio in erythrocyte membranes, BALF cytology, AR to carbachol, and BALF lipoxin A₄ (LXA₄), an endogenous inhibitor of inflammation, were assessed at each time point.

Compared to respective unstimulated values, AS-challenged cats exhibited a significant rise in BALF eosinophil percentage and there was a trend to increased BALF total cell counts, increased AR and reduced BALF LXA₄ concentrations. The significant decrease in the blood ω 6/ ω 3 ratio seen after supplementation demonstrated that ω 3 PUFA were efficiently absorbed. No changes in BALF cytology were found between untreated and treated AS-stimulated cats, but BALF LXA₄ levels were significantly elevated and AR significantly decreased following supplement intake. The study suggests that ω 3-luteolin supplementation may have some beneficial effects on AR through a LXA₄-dependent pathway in cats with experimentally-induced asthma.

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Naturally occurring feline asthma is a chronic respiratory disorder commonly encountered in client-owned cats and, despite the active search for targeting novel and established therapies, its management remains a challenge for practitioners (Hirt, 2005). Lower airway (mainly eosinophilic) inflammation, reversible air-flow limitation and non-specific bronchial hyperresponsiveness are recognised as cardinal features of the clinical condition, and were recently demonstrated in a feline asthma model based on *Ascaris suum* (AS) sensitisation (Kirschvink et al., 2007).

Advances in dietary lipid research have indicated that increased consumption of long chain omega-3 polyunsaturated fatty acids (ω 3 PUFAs) may be of therapeutic value in a variety of acute and chronic inflammatory conditions (Calder, 2006). The ω 6 PUFA arachidonic acid (AA) has emerged as the key link between fatty acids and inflammation since it gives rise to bioactive eicosanoid mediators (prostaglandins, thromboxanes, leukotrienes) and to the subsequent generation of pro-inflammatory cytokines. Not only are long chain ω 3 PUFAs more effective than AA at competing for

eicosanoid synthesis but they also impair inflammatory gene expression and promote anti-inflammatory resolvins and protectins (Calder, 2006).

Multi-pronged benefits of long chain ω 3 PUFAs have been reported in human asthma following treatment with lipid extract of New Zealand green-lipped mussel (GLM) (*Perna canaliculus*), with clinical, functional and inflammatory markers being significantly improved (Emelyanov et al., 2002). Nevertheless, the potential anti-inflammatory properties of ω 3 PUFAs could be counterbalanced by their ability to increase tissue membrane peroxidation. Accordingly, ω 3 PUFAs ingestion should be coupled with concurrent consumption of antioxidants to counteract the oxidative damage of tissue lipids (Song et al., 2000).

The aim of this study was to assess in AS-sensitised and challenged cats the effect of a patented nano-molecular filtered lipid extract from *P. canaliculus* combined with the flavonoid luteolin as antioxidant (BionoVex-L, Bionovate) on airway responsiveness (AR), bronchoalveolar lavage (BAL) cytology and BAL fluid lipoxin A₄ (BALF LXA₄), an endogenous regulator of inflammation.

Eight shorthair AS-sensitised cats aged 28 months (Harlan Netherlands; five neutered males, three neutered females; mean

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weight 4.8 ± 0.4 kg) were enrolled over 3 months in this prospective study, which was approved by the Animal Ethical Committee of the University of Liège. Each cat underwent three protocols successively at 6-week intervals, during which time AR testing, blood sampling and BAL were performed. The first protocol investigated cats with no exposure to allergen for at least 8 months (Unstimulated). After a 6-week recovery period, the cats underwent a single 5-min challenge with an aerosol containing 0.01% of AS to induce an inflammatory response to the allergen (AS-stimulated). The third protocol started at the end of a 4-week dietary supplementation period with similar allergen exposure (Treated + AS-stimulated). The ω 3-luteolin supplement (four tablets/cat/day) provided 20 mg of BionoVex lipids and 10 mg of luteolin. Tablets were crushed and distributed once daily with canned food or with an oral syringe if needed.

AR testing and BAL, respectively, were undertaken 48 h and 72 h following allergenic challenge, as previously described (Kirschvink et al., 2007). AR to inhaled carbachol was defined when the provocative dose increased the enhanced pause ('Penh', a unitless index of bronchoconstriction measured by barometric whole-body plethysmography) to 300% of baseline value (C-Penh₃₀₀, %).

An aliquot of BALF was mixed with gentian violet solution (50:50 vol/vol) and four total nucleated cell counts per sample were performed using a Thoma cell within 4 h of collection. Differential cell counts were carried out on BALF cytospin preparations stained with May–Grünwald–Giemsa. The remaining BALF was centrifuged (450 g) for 10 min at 4 °C and the supernatant stored at –80 °C until analysis for LXA₄. BALF LXA₄ was purified through a C₁₈ 1 mL 100 mg^{–1} column (Bond Elut, Varian) and concentrated 5-fold before quantification by an enzyme immuno-assay kit (Neogen) according to the manufacturer's instructions. Venous blood was sampled by jugular venepuncture into EDTA tubes immediately prior to bronchoscopy. The efficiency of dietary supplementation was assessed by determination of the ω 6/ ω 3 fatty acid ratio in erythrocyte membranes using gas chromatography coupled to flame ionisation detection (GC-FID). Haematology was also monitored at each time point.

Statistic Analysis System (SAS) was used to analyse the data. All variables supported the null hypothesis stating that the data fitted a normal distribution. The random animal effect and fixed effects (i.e. sex and protocols) were investigated with a mixed linear model.

Compared to the respective unstimulated values, AS-challenged cats showed a significant rise in BALF eosinophil % as well as trends towards increased BALF total cell counts, reduced C-Penh₃₀₀ values and BALF LXA₄ concentrations (Table 1). Overall, these allergen-elicited changes were highly suggestive of airway hyperrespon-

siveness and bronchial inflammation – both outcomes being critical for the development of an asthma-like phenotype.

The ω 3-luteolin supplement was well tolerated by all cats and no adverse reactions were reported. Haematology values remained within normal laboratory reference ranges throughout the study (data not shown). As expected, a significant reduction in the calculated ω 6/ ω 3 ratio in erythrocyte membranes was evidenced after supplementation with the ω 3-enriched diet compared to other periods of investigation. Analysis of BALF total and differential cell counts did not reveal any significant differences between treated and untreated AS-stimulated cats, despite a slight decrease in eosinophil % with the dietary supplementation. In contrast, BALF LXA₄ levels and C-Penh₃₀₀ values were significantly enhanced following the supplement intake. Moreover, post-treatment data exceeded those recorded in unstimulated conditions (Table 1).

In horses with recurrent airway obstruction (RAO), 10-week ingestion of seal blubber oil, as a source of long chain ω 3 PUFAs, significantly reduced total cell counts in the pulmonary epithelial lining fluid (Khol-Parisini et al., 2007). Similarly, dietary supplementation with ω 3 PUFAs plus luteolin has been shown to lead to additive decreases in clinical score severity, BALF neutrophil % and levels of exhaled hydrogen peroxide in RAO-affected horses (R. Geering, personal communication). In ovalbumin-sensitised mice, oral treatment with luteolin attenuated an antigen-induced fall in specific airway conductance (SGaw) and bronchial hyperresponsiveness (Das et al., 2003). Nevertheless, it is not widely known whether ω 3 PUFAs and luteolin, alone or in combination, could be beneficial in cats with so-called 'bronchial disease'. In line with previous findings where fish-oil was tested as an ω 3 PUFA supplement (Kirschvink et al., 2006), a 4-week period of ω 3-luteolin treatment prevented an increase in AR in AS-stimulated cats whilst BALF cytology remained broadly unaffected.

In experimentally asthmatic cats sensitised to AS or clinically relevant allergens (Padrid et al., 1995; Norris Reinero et al., 2004; Kirschvink et al., 2007), acute and chronic exposure to allergens caused substantial bronchial hyperresponsiveness in association with eosinophilic influx into airways but no causal relationship between both features of asthma phenotype has been firmly established. A dissociation between airway hyperresponsiveness and eosinophilia has been shown in asthmatic human beings and in animal models of allergic asthma following neutralisation of interleukin (IL)-4 or IL-5 using monoclonal antibodies (Mauser et al., 1993; Corry et al., 1996; Leckie et al., 2000). Eosinophils might not be sufficient or necessary for the development of airway hyperresponsiveness in AS-sensitised and challenged cats. Furthermore, considering the effect of ω 3-luteolin combination upon the activation state of eosinophils (rather than on their sheer number in the

Table 1
Erythrocyte fatty acid levels, airway responsiveness, bronchoalveolar lavage fluid (BALF) concentrations of lipoxin (LX) A₄ and cytological analysis of the BALF in *Ascaris suum*-sensitised cats in remission (Unstimulated), after the first inhaled allergen challenge (AS-stimulated) and after the 4-week dietary supplementation with the combination of ω 3 polyunsaturated fatty acids (PUFAs) and flavonoid luteolin (Treated + AS-stimulated).

Parameters	Units	Unstimulated cats (n = 8)	AS-stimulated cats (n = 8)	Treated + AS-stimulated cats (n = 8)
ω 3 PUFA	Mole %	1.68 ± 0.20^a	1.59 ± 0.17^a	2.22 ± 0.42^b
ω 6 PUFA	Mole %	42.8 ± 0.7^a	41.5 ± 0.5^b	43.7 ± 0.7^c
ω 6/ ω 3	–	25.7 ± 3.3^a	26.4 ± 3.0^a	20.2 ± 3.4^b
C-Penh ₃₀₀	% (g/100 mL)	0.025 ± 0.012^{ab}	0.021 ± 0.012^a	0.029 ± 0.012^b
LXA ₄	pg/mL	62.2 ± 10.2^{ab}	36.8 ± 12.2^a	67.2 ± 34.9^b
Total cells	$\times 10^3$ cells/mL	187 ± 120	290 ± 186	267 ± 214
Macrophages	%	83.8 ± 7.2^a	44.1 ± 20.4^b	55.7 ± 19.4^b
Neutrophils	%	9.0 ± 6.1	10.5 ± 6.7	7.6 ± 3.1
Eosinophils	%	7.2 ± 3.3^a	43.6 ± 18.6^b	36.1 ± 16.9^b

Data are expressed as means $\pm$ SD (standard deviation). ^{a,b,c}Data with different superscripts are significantly different, $P < 0.05$.

AS, *Ascaris suum*; ω 3 and ω 6 PUFA, total omega-3 and omega-6 PUFAs; ω 6/ ω 3, ratio between total omega-3 and omega-6 PUFAs; C-Penh₃₀₀, carbachol concentration inducing a 300% increase of baseline Penh (enhanced pause); LXA₄, lipoxin A₄.

airways) might provide an alternative explanation for our results. Lefort et al. (1996) demonstrated the requirement of local eosinophil activation and the subsequent release of their granule-derived products in achieving bronchial hyperresponsiveness in actively sensitised guinea-pigs. In our model, airway responsiveness decreased after ω 3-luteolin treatment whilst eosinophilic inflammation did not, and the cellular and molecular mechanisms underlying these divergent observations still remain uncertain.

Besides modulating bronchial responsiveness, the ω 3-luteolin supplement significantly enhanced the local generation of LXA₄. Lipoxins are biologically active eicosanoids biosynthesised in vivo at inflammation sites to mediate the resolution of inflammatory processes (Levy, 2005). Differentiated human bronchial epithelial cells produce LXA₄ in vivo and this significantly inhibits tumour necrosis factor (TNF)- α -induced IL-8 release through autocrine action on its specific receptor, named formyl-peptide receptor like-1 (Bonnans et al., 2007). Potential benefits for asthma have been seen using a murine experimental model where IV administration of a LXA₄ analogue prior to ovalbumin challenge decreased pulmonary infiltration of eosinophils and lymphocytes, along with attenuated airway hyperresponsiveness (Levy et al., 2002). Interestingly, untreated AS-stimulated cats displayed lower LXA₄ concentrations in BALF supernatant compared to controls. Depleted levels of LXA₄ in supernatants of induced sputum (Bonnans et al., 2002; Vachier et al., 2005) and BALF (Planaguma et al., 2008) or in activated whole blood (Celik et al., 2007) were detected in severe asthmatic patients in contrast to other severity groups (mild-moderate) or healthy subjects, suggesting LXA₄ production deficiency. Thus, increased LXA₄ synthesis (as in treated cats) might exert a protective role against airway hyperresponsiveness.

Our results raise the question as to how exogenous ω 3 PUFAs triggered LXA₄ generation. Lipoxins are produced from AA (ω 6 PUFA) during cell-cell interactions at sites of inflammation. Through the action of airway epithelial 15-lipoxygenase (15-LO), AA is initially transformed into 15S-hydroxy-eicosatetraenoic acid which can serve as substrate for further conversion to LXA₄ by 5-LO from activated leukocytes circulating in inflamed airways (Levy, 2005). However, given the ω 3-enriched composition of our treatment, this biosynthetic scheme is unlikely to be relevant. Aspirin is now well recognised for its ability to elicit formation of 15R-epimers of LX (carbon-15 alcohol in the R configuration), specifically termed 15-epi-LXA₄ and 15-epi-LXB₄. They share the potent actions of the native 15S-containing forms and arise from cooperation between aspirin-acetylated cyclooxygenase-2 (COX-2) of epithelial cells and leukocyte 5-LO (Levy, 2005). The lipid extract from GLM has been shown to downregulate the COX-2 pathway in activated human macrophages in vitro (Whitehouse et al., 1997), and therefore might promote 15-epi-LXA₄ biosynthesis. As argued above, whether or not ω 3 PUFAs per se initiate generation of LXA₄ is not yet clear, and a deeper knowledge of GLM lipid extract composition should elucidate mechanisms supporting our findings.

A limitation of the present trial was the lack of cross-over evaluation of the effects of supplementation on allergen challenge in the same cat. However, earlier studies of our group have shown that monthly AS-challenges in cats induced very similar patterns of asthmatic response (Kirschvink et al., 2007). Even if these were significant, treatment-related changes in total ω 3 PUFAs were of lesser magnitude than those reported in healthy cats supplemented with docosahexaenoic acid-enriched salmon oil for 4 weeks (Filburn and Griffin, 2005). It may be speculated that discrepancies between both studies are possibly related to differences in experimental setting (i.e. the time interval between the last administration of dietary supplement and the blood sampling for fatty acid analysis) and chemical composition of the ω 3-enriched diet. Finally, it needs to be noted that this trial was conducted on

experimental cats with acute allergic inflammation and our results might therefore not be reflective of alterations in chronically asthmatic pet cats. In human asthmatics, ω 3 PUFA supplementation led to decreases in the production of the 4-series leukotrienes and some pro-inflammatory cytokines (e.g. TNF- α , IL-1 β) without consistent improvement in clinical outcomes (Wong, 2005).

In AS-sensitised cats, allergen-induced changes in AR could be partially modulated through a LXA₄-dependent pathway by oral administration of a lipid extract of New Zealand GLM and luteolin combination. Further investigations on experimental or client-owned asthmatic cats should help to determine the significance of these anti-inflammatory effects.

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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