

Assessment of circulating concentrations of proinflammatory and anti-inflammatory cytokines and nitric oxide in dogs with brachycephalic airway obstruction syndrome

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Objective—To evaluate plasma concentrations of inflammatory mediators in dogs with brachycephalic airway obstruction syndrome, identify a possible role for these mediators in the syndrome, and investigate the relationship between plasma concentrations of inflammatory mediators and severity of clinical signs.

Animals—17 dogs with brachycephalic airway obstruction syndrome and 10 mesocephalic (control) dogs.

Procedures—A blood sample was collected once from each dog. Plasma concentrations of interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , IL-6, IL-17A, IL-10, and IL-13 were measured with ELISAs. Nitric oxide (NO) concentrations were determined with a Griess test. For analysis, brachycephalic dogs were categorized into groups depending on weight (small [< 16 kg]) and large [≥ 16 kg]) or on whether they required medical or surgical treatment.

Results—Compared with control dog values, plasma concentrations of TNF- α , IL-10, IL-13, and IL-17A were significantly higher in brachycephalic dogs and markedly so for brachycephalic dogs that required surgery; findings for small and large brachycephalic dogs did not differ. A similar pattern of differences between control and brachycephalic dogs was identified for plasma NO concentration. Plasma IL-1 β and IL-6 concentrations in control and brachycephalic dogs did not differ.

Conclusions and Clinical Relevance—In brachycephalic dogs, plasma TNF- α , IL-10, IL-13, IL-17A, and NO concentrations were higher than values in control dogs and appeared to be associated with disease severity. These variables may be useful as indicators of inflammatory processes associated with brachycephalic airway obstruction syndrome in dogs. (*Am J Vet Res* 2013;74:155–160)

An upper respiratory tract disease known as BAOS has been identified in brachycephalic dogs since the beginning of the 20th century. The syndrome is comprised of morphological and functional alterations involving the upper airways, lower airways, and digestive system.^{1–4} Breeds commonly affected include English Bulldog, French Bulldog, Pug, and Boston Terrier, although BAOS has also been detected in Pekingese, Cavalier King Charles Spaniel, Shih Tzu, Lhasa Apso, Boxer, and Shar-Pei. Both sexes are affected. Treatment

ABBREVIATIONS	
BAOS	Brachycephalic airways obstruction syndrome
IL	Interleukin
NO	Nitric oxide
TNF	Tumor necrosis factor

for brachycephalic airways obstruction syndrome can be surgical^{3–12} or medical. However, medical management may only temporarily improve clinical signs and provide palliation, which can delay definitive treatment and result in progression of the disease to an advanced stage.¹³

In advanced stages of BAOS, secondary changes such as tonsillar hypertrophy, everted saccules, and laryngeal collapse develop as a result of airflow turbulence during respiratory cycles. These changes dramatically reduce airway diameter, leading to a marked worsening of the respiratory obstruction. Severe upper airway obstruction can result in development of pulmonary edema because of a reduction in intrathoracic pressure. Inadequate pulmonary ventilation caused by upper air-

Received December 12, 2011.

Accepted April 3, 2012.

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Presented in an abstract form at the 20th Annual Scientific Meeting of the European College of Veterinary Surgeons, Ghent, Belgium, July 2011.

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way obstruction can lead to a reduction of blood oxygen content and subsequent hypoxia. Hypoxia may also increase fatigability of upper airway dilator muscle and soft tissue in a manner that has the potential to worsen the disease, as indicated by results of a study¹⁴ in adult rats. Furthermore, respiratory obstruction is a risk factor for suffocation during anesthesia and the postsurgical period.¹⁵ Inflammation as well as oxidative and nitrosative stress associated with repeated hypoxia and reoxygenation could be implicated in the pathogenesis of BAOS; however, despite the fact that anatomic and functional alterations are well described,^{16–23} the molecular mechanisms involved in this syndrome remain unknown. To our knowledge, there are no reports of the molecular mediators implicated in BAOS. Thus, the objectives of the study reported here were to evaluate plasma concentrations of inflammatory mediators in dogs with BAOS, identify a possible role for these mediators in the syndrome, and investigate the relationship between plasma concentrations of inflammatory mediators and severity of clinical signs. We hypothesized that a proinflammatory condition could be related to the pathogenesis and the progression of BAOS.

Materials and Methods

Animals—Other than the control dogs, only purebred brachycephalic dogs with BAOS were included in the study. At admission, each dog's case history was obtained from the owner, focusing on questions about the severity of stertor, stridor, or snoring; degree of exercise intolerance; presence of syncope or cyanosis; and gastrointestinal signs, such as vomiting, regurgitation, and signs of nausea. A clinical examination was then performed, with special emphasis on patency and symmetry of the nares, careful palpation of larynx and tracheal rings, and auscultation of the heart and lung fields. By combining the information obtained from the owners and that obtained during the clinical examination, a score was assigned to each brachycephalic dog on the basis of the scoring system proposed by Poncet et al.²³ Briefly, the frequency of 4 upper respiratory tract signs (ie, snoring, inspiratory effort, exercise or stress intolerance, and syncope) was each assessed; on the basis of the frequency of each respiratory sign in combination, a global grade classification was obtained for each dog: not affected (grade 1), moderately affected (grade 2), or severely affected (grade 3). A complete hematologic and biochemical analysis was performed for each brachycephalic dog to rule out systemic diseases. Dogs with potential systemic disease (eg, heart, liver, or kidney failure) were excluded from the study. Similarly, dogs with aggressive or particularly excitable behaviors were excluded from the study.

For purposes of data analysis, brachycephalic dogs were categorized into 2 groups on the basis of weight: small (< 16 kg) and large (≥ 16 kg). The brachycephalic dogs were also categorized into 2 groups on the basis of whether they required immediate surgical treatment or received medical treatment. Dogs that underwent medical treatment were further categorized (depending on results of clinical examination) into 3 groups: those with no clinical signs, moderate clinical signs, or severe clinical signs. Ten clinically normal 2- to 3-year-

old Beagles (5 males and 5 females) were used as the control group.

The study was approved by the Complutense University Ethical Committee and adhered to the guidelines of Commission Directive 86/609/EEC (The Council Directive of the European Community) concerning the protection of animals used for experimental and other scientific purposes. The national legislation, in agreement with this directive, is defined in Royal Decree No. 1201/2005.

Preparation of samples—A blood sample (10 mL) was collected from a cephalic vein of each dog. The samples were centrifuged at 3,000 × g for 10 minutes at 4°C, and the plasma was transferred to polypropylene tubes and frozen at –80°C until assayed.

Determinations of plasma cytokine concentrations—Concentrations of proinflammatory (IL-1β, TNF-α, IL-6, and IL-17A) and anti-inflammatory (IL-10 and IL-13) cytokines were measured in plasma samples with specific ELISA commercial kits^a according to the manufacturer's protocols. Briefly, plasma samples and standard dilutions of the tested cytokine were incubated onto special microwells coated with specific monoclonal antibodies. The sample incubation was performed at 18° to 25°C for 1 to 3 hours, depending on the specific cytokine and the manufacturer's instructions. A second antibody and a horseradish peroxidase conjugate were added afterward, followed by incubation for 30 minutes at 18° to 25°C.

Plates were developed with 3,3',5,5'-tetramethylbenzidine as a chromogen substrate; 12 to 15 minutes later, the reaction was stopped with a sulfuric acid solution and plates were evaluated at 450 nm. Each cytokine concentration was quantified against a standard curve.

Determination of plasma NO concentration—Plasma NO concentration was measured by the Griess reaction (ie, NO₂ concentration was determined after the reduction of NO₃ to NO₂). Plasma samples were deproteinized by the addition of sulfosalicylic acid, incubated for 30 minutes at 4°C, and subsequently centrifuged (12,000 × g for 20 minutes). After incubating the supernatants with *Escherichia coli* NO₃ reductase (at 37°C for 30 minutes), 1 mL of Griess reagent (0.5% naphthylendiamine dihydrochloride, 5% sulfonilamide, and 25% H₃PO₄) was added. The reaction was allowed to proceed for 20 minutes at 22°C, and the absorbance at 546 nm was measured; an NaNO₂ solution was used as a standard. The measured signal was linear from 1 to 150 μM ($r = 0.994$; $P < 0.001$; $n = 5$), and the detection threshold was approximately 2 μM.

Reproducibility within each assay was evaluated in 3 independent experiments, and each assay was performed with 3 replicates. The overall intra-assay coefficient of variation was calculated to be < 5%. Interassay reproducibility was evaluated in 3 independent experiments. The overall interassay coefficient of variation was calculated to be < 6%.

Statistical analysis—Results are expressed as the mean ± SEM. Significant differences in plasma cytokine and NO concentrations between the control and

brachycephalic dogs, between the control and small or large brachycephalic dogs, and among all groups (control dogs, brachycephalic dogs with no clinical signs, moderate clinical signs, or severe clinical signs and brachycephalic dogs that underwent surgery) were determined with the nonparametric Kruskal-Wallis test and subsequently with the Mann-Whitney *U* test. A confidence level of 95% and values of *P* < 0.05 were considered significant.

Results

Dogs—Twenty-one brachycephalic dogs were considered for the present study. Three of the dogs were excluded from the study because they had a systemic illness, and 1 was excluded because it had an excitable character. Seventeen brachycephalic dogs were included in the study, of which 9 were sexually intact males, 6 were sexually intact females, and 2 were spayed females. The median age of the brachycephalic dogs was 3.3 years (range, 11 months to 7 years). All were pure-bred dogs. English (*n* = 8) and French (4) Bulldogs were the most common breeds. For purposes of data analysis, the brachycephalic dogs were categorized into 2 groups on the basis of weight: 7 dogs were considered small (< 16 kg), and 10 dogs were considered large (≥ 16 kg). The brachycephalic dogs were also categorized into 2 groups on the basis of whether they required immediate surgical treatment (*n* = 4) or received medical treatment (13). Dogs that underwent medical treatment were further categorized into 3 groups: those with no clinical signs (*n* = 6), moderate clinical signs (5), and severe clinical signs (2). The control group included 10 clinically normal 2- to 3-year-old university-owned Beagles (5 males and 5 females; all were sexually intact).

Plasma concentrations of proinflammatory cytokines—In the 17 brachycephalic dogs, mean plasma concentration of TNF- α was significantly higher than

the value in the control dogs (Table 1). Mean plasma TNF- α concentration was significantly higher in both small and large brachycephalic dogs, compared with the value in the control dogs. Brachycephalic dogs with no clinical signs had significantly higher mean plasma TNF- α concentration than did control dogs; this difference was more pronounced in brachycephalic dogs with severe clinical signs and in those that required immediate surgical treatment.

Mean plasma concentration of IL-17A in the 17 brachycephalic dogs, small brachycephalic dogs, or large brachycephalic dogs did not differ significantly from the value in the control dogs. However, mean plasma IL-17A concentration was significantly higher in both brachycephalic dogs with severe clinical signs and those that required immediate surgical treatment, compared with values in the control dogs, brachycephalic dogs with no clinical signs, and brachycephalic dogs with moderate clinical signs (Table 1). Mean plasma concentrations of IL-1 β and IL-6 in the control dogs and the 17 brachycephalic dogs did not differ. Mean plasma IL-6 concentration in brachycephalic dogs with moderate clinical signs was significantly less than the value in the control dogs; however, there were no significant differences identified in any other group comparisons.

Plasma concentrations of anti-inflammatory cytokines—In the 17 brachycephalic dogs, mean plasma concentration of IL-10 was significantly higher than the value in the control dogs (Table 1). Mean plasma IL-10 concentration was significantly higher in both small and large brachycephalic dogs, compared with the value in the control dogs. Brachycephalic dogs with moderate clinical signs, those with severe clinical signs, and those that required immediate surgical treatment had significantly higher plasma IL-10 concentrations, compared with the value in the control dogs.

Table 1—Mean \pm SD plasma concentrations of proinflammatory and anti-inflammatory cytokines and NO determined at the time of initial evaluation in 10 mesocephalic dogs (controls) and 17 brachycephalic dogs with BAOS that were further categorized on the basis of weight (small [*<* 16 kg] or large [*≥* 16 kg]) and whether they required immediate surgical treatment or received medical treatment.

Group	Proinflammatory cytokine				Anti-inflammatory cytokine		NO (nmol/mL)
	TFN- α (pg/mL)	IL-1 β (pg/mL)	IL-6 (pg/mL)	IL-17A (pg/mL)	IL-10 (pg/mL)	IL-13 (pg/mL)	
Control (<i>n</i> = 10)	35.95 \pm 11.23	98.06 \pm 10.89	24.12 \pm 7.96	37.40 \pm 9.53	12.40 \pm 0.90	1.63 \pm 0.34	77.23 \pm 12.12
Brachycephalic							
All (<i>n</i> = 17)	84.61 \pm 11.04*	105.44 \pm 13.17	21.38 \pm 6.02	38.96 \pm 9.73	18.77 \pm 1.39*	2.72 \pm 0.76*	95.73 \pm 12.22*
Small (<i>n</i> = 7)	93.22 \pm 23.83*	87.75 \pm 6.61	14 \pm 3.63	58.84 \pm 24.93	19.77 \pm 1.94*	2.44 \pm 0.51*	98.11 \pm 10.53*
Large (<i>n</i> = 10)	75.70 \pm 10.39*	102.92 \pm 6.04	28.76 \pm 11.30	49.85 \pm 18.43	17.46 \pm 2.05*	2.36 \pm 0.51*	99.33 \pm 13.9*
Surgery required (<i>n</i> = 4)	121.55 \pm 13.76*	81.92 \pm 8.52	18.08 \pm 4.53	127.27 \pm 0.76*	19.32 \pm 2.54*	2.04 \pm 0.45	114.98 \pm 9.83*
Medical treatment							
No clinical signs (<i>n</i> = 6)	82.62 \pm 18.74*	81.51 \pm 10.19	16.64 \pm 6.65	28.85 \pm 4.1	14.14 \pm 1.85	2.79 \pm 0.61*	76.77 \pm 7.71
Moderate clinical signs (<i>n</i> = 5)	84.14 \pm 10.21*	108.55 \pm 7.16	11.43 \pm 2.52*	40.36 \pm 17.97	20 \pm 1.51*	1.63 \pm 0.42	89.89 \pm 5.1
Severe clinical signs (<i>n</i> = 2)	107.42 \pm 16.10*	86.84 \pm 6.96	22.40 \pm 4.1	125.57 \pm 1.25*	20.54 \pm 2.40*	1.88 \pm 0.41	108.92 \pm 9.43*

Dogs that underwent medical treatment were further categorized (depending on results of clinical examination) into 3 groups: those with no clinical signs, moderate clinical signs, or severe clinical signs.
*Within a variable, value is significantly (*P* < 0.05) different from the value for the control dogs.

Mean plasma concentration of IL-13 in the 17 brachycephalic dogs, small brachycephalic dogs, or large brachycephalic dogs was significantly higher than the value in the control dogs (Table 1). Mean plasma IL-6 concentration in brachycephalic dogs with no clinical signs was significantly higher than the value in the control dogs; however, there were no significant differences identified in any other group comparisons.

Plasma concentration of NO—In the 17 brachycephalic dogs, mean plasma concentration of NO was significantly higher than the value in the control dogs (Table 1). Mean plasma NO concentration was significantly higher in both small and large brachycephalic dogs, compared with the value in the control dogs. Although there was a progressive increase in mean plasma NO concentration in brachycephalic dogs with increasing severity of clinical signs, compared with the value in the control dogs, the difference was significant only for brachycephalic dogs with severe signs. Brachycephalic dogs that required immediate surgical treatment had significantly higher plasma NO concentration, compared with the value in the control dogs.

Discussion

Brachycephalic airway obstruction syndrome is a serious and potentially deadly combination of upper airway disorders in predisposed breeds. In the present study, 17 brachycephalic dogs were evaluated, 12 of which were Bulldogs. This study group was not representative of all brachycephalic breeds or the breeds that are commonly affected with BAOS, which was a limitation of this investigation. In the present study, we hypothesized that a proinflammatory condition could be related to the pathogenesis and the progression of BAOS.

Inflammation is a complex host's normal defense reaction to insult and stress. Inflammation responses, whether acute or chronic, are activated by well-coordinated, sequential events that are controlled by humoral and cellular reactions. Cytokines are the major communication channels that provide links within and between the immune system and other organs. An inflammatory response is associated with a spontaneous increase in concentrations of proinflammatory cytokines; those that have obtained most attention are IL-1 β , IL-6, and TNF- α . In the present study, plasma TNF- α concentration in brachycephalic dogs was significantly higher than the value in clinically normal nonbrachycephalic control dogs; this significant difference in plasma TNF- α concentration was apparent between control dogs and both small (weight < 16 kg) and large (weight \geq 16 kg) brachycephalic dogs. These findings agree with results of studies²⁴⁻²⁷ performed in other species, including humans, which identified an association between the concentration of TNF- α in plasma and advanced stages of respiratory obstructive disease. The possible relationship between plasma concentrations of TNF- α and the degree of BAOS disease in dogs has not been described.

Tumor necrosis factor- α is a pivotal proinflammatory cytokine, which is centrally involved in local and

systemic responses in the immune system that lead to typical effects of inflammation. Unregulated, prolonged synthesis and release of TNF- α in chronic inflammatory conditions may contribute to the development of many diseases. Tumor necrosis factor- α can directly induce tissue injury by inducing accelerated cell apoptosis but may also initiate and perpetuate an inflammatory response through upregulation of the expression of other inflammatory mediators. It is known that the TNF- α triggers a cascade of cytokines and angiogenic factors that contribute to endothelial changes first within the lung parenchyma and then systemically.^{27,28} However, these effects have never been investigated in relation to BAOS, to our knowledge. In the study reported here of brachycephalic dogs with BAOS, plasma TNF- α concentration was significantly higher in dogs with no clinical signs, compared with unaffected control dogs; this difference was more pronounced in brachycephalic dogs with severe clinical signs and in those that required immediate surgical treatment, suggesting that TNF- α could play an important role in the pathogenesis of BAOS.

Because of its ability to stimulate the production of other proinflammatory cytokines and activate nuclear factor- κ B, TNF- α could contribute to the maintenance and exacerbation of ongoing inflammatory processes. This increase in chronic inflammatory process may cause changes of the lung parenchyma that lead to pulmonary hypertension, respiratory failure, or cardiac dysfunction. These pulmonary or cardiac alterations are often present in advanced stages of BAOS, suggesting an important role of inflammation in their development. In the present study, grade 1 and grade 2 brachycephalic dogs had significantly higher plasma TNF- α concentrations, compared with control dogs; therefore, it is possible that even in the early stages of BAOS, there could be alterations in the lung parenchyma (allowing passage of mediators to the bloodstream), implying the presence of systemic disorders. Increases in circulating concentrations of TNF- α are often accompanied by increases in concentrations of IL-1 β and IL-6; however, in the present study, plasma IL-1 β and IL-6 concentrations were not altered in dogs with BAOS, compared with values in the control dogs.

Interleukin-17 has been characterized as a proinflammatory cytokine that acts on a variety of tissues. In chronic inflammatory processes associated with the respiratory tract in humans, such as allergic asthma, IL-17A, IL-17F, and IL-17E have been demonstrated to have an important role.^{29,30} Findings of those studies^{29,30} suggest that IL-17A and IL-17F are involved in subepithelial fibrosis that leads to airway remodeling, a process related to the severity of the disease. Results of the present study seem to be in agreement, at least partially, with those of the aforementioned studies.^{29,30} In fact, plasma IL-17A concentration was significantly higher in both brachycephalic dogs with severe clinical signs and those that required immediate surgical treatment, compared with the value in control dogs. This could be explained by hypothesizing that IL-17A acts in the later stages of the disease and promotes vascular remodeling, which in turn would then cause hypertension typical of advanced stages of obstructive respiratory syndromes.

The mechanism of signaling and activation of IL-17A has not been fully defined.³¹ Therefore, it is difficult to understand the role of IL-17A in obstructive respiratory syndromes, especially in veterinary medicine, wherein the only study³² of this proinflammatory cytokine, to our knowledge, investigated its role in osteoarthritis in canine hip joints.

Interleukin-10 is regarded as an excellent immunosuppressive cytokine. It acts on macrophages by suppressing the secretion of proinflammatory cytokines and promoting the production of cytokine inhibitors. Its contribution to the resolution of airway inflammatory processes³³ and its anti-inflammatory activity in chronic obstructive pulmonary disease³⁴ have been demonstrated. This cytokine is so important for the control and resolution of obstructive respiratory syndromes, it has been proposed in human medicine as a marker for monitoring chronic obstructive pulmonary disease and for use in the treatment of the disease (as well as other nonsteroidal drugs, such as inhibitors of phosphodiesterase-4 and inhibitors of nuclear factor- κ B³⁵).

Interleukin-13 has immunosuppressive activity together with IL-4 and IL-10 and inhibits production of inflammatory cytokines by monocytes. It also increases the proliferation and differentiation of monocytes and B cells. Data from several studies have highlighted the role of IL-13 in the pathogenesis of respiratory tract diseases (eg, asthma and chronic obstructive pulmonary disease)³⁶ and established a direct association between plasma concentrations of this cytokine and degree of disease.³⁷ In the study reported here, both plasma IL-10 and IL-13 concentrations were significantly higher in brachycephalic dogs, compared with values in control dogs, which suggested that anti-inflammatory cytokines could play an important role in the pathogenesis of BAOS. On the other hand, reactive nitrogen species are heavily implicated in the inflammatory process. Excessive NO production by inducible NO synthase may have an important role in tissue injury secondary to an inflammatory response. In the present study, plasma concentrations of nitrate and nitrite (the stable end products of NO) were assessed to estimate the difference in NO production between brachycephalic and control dogs. The plasma concentrations of NO were significantly increased in brachycephalic dogs, compared with the value in control dogs. Plasma concentrations of NO increased progressively in brachycephalic dogs with no, moderate, and severe clinical signs, compared with control dog findings; plasma NO concentrations in brachycephalic dogs with severe clinical signs and those that required immediate surgical treatment were significantly greater than the value in control dogs. A similar pattern of increasing increase plasma TNF- α concentration with worsening clinical signs of BAOS was identified, suggesting the existence of a possible association between the production of NO and the expression of some cytokines. This association may underlie the regulation of the disease process, increasing the inflammatory response and thus contributing to the development of secondary changes.

Results of the present study have suggested that BAOS is not, as hitherto considered, a local process but

rather a set of changes at the local level that result in systemic pathological changes through the production of inflammatory mediators. An improved understanding of the mechanisms involved in worsening BAOS may aid in the identification of dogs at risk for disease progression and lead to alternative treatments that could prevent development of secondary changes.

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