Nerve growth factor concentrations in the synovial fluid from healthy dogs and dogs with secondary osteoarthritis

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Keywords
Nerve growth factor, synovial fluid, canine osteoarthritis, lameness, pain

Summary
Objective: To measure the concentrations of nerve growth factor (NGF) in the synovial fluid from normal dogs and dogs with osteoarthritis (OA) secondary to common joint disorders.

Methods: Nerve growth factor synovial concentrations were measured by ELISA assay in 50 dogs divided into three groups: 12 healthy, 16 affected by acute lameness within seven days before enrolment, and 22 with chronic lameness persisting by more than one month before enrolment and accompanied by radiological signs of OA. Both acute and chronic lameness were secondary to orthopaedic diseases involving the shoulder, elbow and stifle joints. Nerve growth factor synovial concentrations were compared between means for healthy and acute groups and between the three groups using an F-test. Significance level was set at p < 0.05.

Results: Nerve growth factor was detected in all canine synovial fluid samples. However, the mean synovial NGF concentration of healthy dogs (3.65 ± 2.18 pg/ml) was not significantly different from the mean value in dogs with acute lameness (6.45 ± 2.45 pg/ml) (p = 0.79). Conversely, the mean synovial NGF concentration in dogs with chronic lameness (20.19 ± 17.51 pg/ml) was found to be significantly higher than that found in healthy dogs (p < 0.01).

Clinical significance: This study demonstrates for the first time the presence of NGF in canine synovial fluid and its increased concentrations in dogs with chronic lameness compared to healthy dogs and dogs with acute lameness. The association between chronic lameness and raised synovial concentrations may suggest an involvement of NGF in OA inflammation and chronic pain.

Introduction
Osteoarthritis (OA) is one of the most common chronic musculoskeletal diseases in dogs, affecting 20% of the canine population over one year of age (1). The development of OA is mainly secondary to trauma, joint instability, and diseases such as hip dysplasia (2). Osteoarthritis is a disease condition of the entire joint, and both inflammatory and degenerative changes of all articular structures result in disability and clinical signs of lameness and pain (2). Pain is the most important clinical manifestation of canine OA and it is the result of a complex interplay between structural joint changes, biochemical and molecular alterations, as well as peripheral and central pain-processing mechanisms (2). Within this intriguing network, the activation and sensitization of peripheral nociceptors by inflammatory and hyperalgesic mediators (e.g. cytokines, prostaglandins and neuromediators) is one of the main peripheral mechanisms responsible for the joint pain (3). Nerve growth factor (NGF) is one of the neuromediators that has received broader attention as a key regulator involved in both inflammatory and neuropathic pain (4, 5). Nerve growth factor is the best characterized member of the family of neurotrophins, and it was first isolated in the early 1950s by the Nobel prize-winner Rita Levi-Montalcini (6, 7). Nerve growth factor-induced signals are mediated by two distinct cell surface receptors, respectively named the high-affinity receptor TrkA (tyrosine kinase receptor) and the p75 low-affinity receptor (8). Although originally discovered as a trophic factor for sensory and sympathetic neurons, it now appears that NGF is a key factor in neuro-immune, inflammatory and nociceptive tissue responses (8–12). Indeed, the concentrations of NGF rise in many acute and chronic pain conditions (4). In particular, it has been well documented that: (i) basal concentrations of NGF rapidly increase in several allergic, autoimmune and chronic inflammatory disorders (13, 14); (ii) NGF synthesis can be induced by pro-inflammatory cytokines such as interleukins 1 and 6 and tumour necrosis factor (TNF) (15); (iii) NGF is produced and released at sites of injury and inflammation by different non-neural cell types, includ-
ing mast cells, which also express NGF receptors (16, 17). Finally, a relationship has been suggested between the increased NGF concentrations, the severity of the inflammatory process, and the increased numbers of NGF-responsive cells (e.g. mast cells) at sites of inflammation and nerve injury (11, 18). Taken together, these findings depict the pleiotropic nature of NGF, which is now viewed as a cytokine-like neuromediator (neurokine) involved in the integrated neuro-immune adaptive response to different offending stimuli and stressful events, as far as in pathophysiology of inflammatory and nociceptive processes (8, 11, 18). The fact that NGF shows such a large spectrum of actions raised the question if, and to what extent, this neurotrophin could be involved in the pathophysiology of joint inflammation and pain (19). So far, increased concentrations of NGF have been reported in the synovial fluid and tissues from inflammatory and autoimmune arthritides when compared to non-inflammatory controls both in humans and animal models (20–22). Furthermore, it has been shown that the increased concentrations of NGF in synovial fluid are also associated with the presence of inflammatory cytokines and increased local expression of NGF high-affinity receptor system (23, 24). Finally, there is also evidence that NGF is directly related to hyperalgesia and pain in animal models of autoimmune arthritis, and that the treatment with NGF antagonists, as well as with NGF-neutralizing molecules, results in a rapid and sustained reduction of moderate to severe knee osteoarthritic pain (20, 25, 26). Recent studies have been performed using preclinical models of OA, but as far as we know there is not any published clinical research evaluating the synovial concentrations of NGF in the dog (5). Therefore, in order to better understand the potential involvement of this neurokine in canine joint pain and inflammation, we determined the concentrations of NGF in the synovial fluid of normal dogs and dogs with secondary OA that resulted from various joint disorders.

**Materials and methods**

**Sample population**

Fifty client-owned dogs of various breeds and ages were chosen from the patients presented at the Department of the Veterinary Clinical Sciences, University of Padua (Italy). The study’s protocol was approved by the local Ethics Committee, and informed owner consent was obtained prior to participation in this study. Dogs which were presented to the clinical institution for routine medical examination, or to be neutered (spaying, castration), and which did not have any history of orthopaedic abnormalities, were included in the healthy control group, Group A. One stifle or elbow from each of these dogs was selected at random, and radiographic images with orthogonal views in a cranio-caudal and a medio-lateral direction were obtained. All radiographs were performed with the animals sedated (methadone 0.2 mg/kg/IM and acepromazine 0.02 mg/kg/IM). The radiographs were examined to ensure that the joints were free of any radiographic signs of OA or other orthopaedic abnormalities. Dogs with a history of neurological, immune-mediated or musculoskeletal diseases, or those that had undergone orthopaedic surgery within one year were excluded. Dogs that were presented by their owners with the complaint of lameness, and which were diagnosed as suffering from a joint disease after complete orthopaedic evaluation and clinical gait examination were recruited to the study. Radiographic examination of the affected joints was performed in all dogs as described before. Dogs with lameness of less than seven days were included in Group B, unless they had any radiographic sign of chronicity. Dogs with lameness of greater than one month duration and radiographic evidence of osteophytes, subchondral bone sclerosis, or both, were included in Group C. All dogs were otherwise healthy. Dogs that underwent orthopaedic surgery on the affected joint were excluded. None of the dogs had received non-steroidal anti-inflammatory drugs or other anti-inflammatory medications in the two weeks prior to the inclusion.

**Collection of synovial fluid samples**

A sample of synovial fluid was collected by arthrocentesis from one joint of each dog (stifle or elbow from the control Group A animals; affected shoulder, elbow or stifle in the acute Group B and chronic Group C). Briefly, the joint region was prepared by shaving and scrubbing. After sedation with intravenously administered medetomidine (0.01 ml/kg/body weight), synovial fluid was aseptically aspirated from the designated joint using a 22-gauge or 20-gauge needle and a 3 ml syringe. Synovial fluid samples of at least 500 μL were considered acceptable for use in the study.

**Preparation of synovial fluid samples**

Synovial fluid specimens from all groups of dogs were immediately centrifuged at 10,000 g for 20 min at 4°C to remove any cell and debris, and supernatants were carefully removed and stored at –80°C until used for the NGF immunoenzymatic assay.

**Nerve growth factor enzyme-linked immunosorbent assay**

The concentrations of NGF in synovial supernatants were measured using antibody-based, sandwich enzyme-linked, immunosorbent assay (ELISA) kit strictly according to the procedures recommended by the manufacturer. Briefly, the assay was a two-site sandwich ELISA that offers optimal sensitivity as low as 10–15 pg/ml, with a range of detection of 10–1000 pg/ml and no significant cross-reactivity with other neurotrophins (<2%). The 96-well microplate provided in the kit, which had been pre-coated with a sheep polyclonal antibody specific for NGF, was used to capture the soluble NGF from all samples (‘capturing’ antibody). During the second incu-

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a Domitor®: Pfizer Animal Health, Exton, PA, USA
b Chemikine™ NGF Sandwich ELISA Kit, cat. no. CYT304: Chemicon International, Inc., Temecula, CA, USA
bation step, a NGF-specific, biotin-conjugated mouse monoclonal antibody detected the captured NGF (‘detecting’ antibody). In a third incubation step, a standardized preparation of horse radish peroxidase (HRP)-conjugated mouse anti-IgG polyclonal antibody detected the anti-NGF mouse monoclonal antibody. After addition of a streptavidin-enzyme conjugate, in order to remove any unbound antibody-enzyme reagent, a 3,3’5,5’ tetramethyl-benzidine colourburst substrate solution was added to each well and the colourimetric amount of NGF bound in the initial incubation step was determined. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the colour change evaluated using a spectrophotometer© with a 450 nm reading filter and a 630 nm reference filter. For each single plate, a linear regression curve was obtained based on the standard curve optical density (OD) values (Fig. 1). The standard curve, which was generated by plotting the average OD (450 nm) obtained for each of the standard concentrations on the Y-axis versus the corresponding NGF concentration (pg/ml) on the X-axis, demonstrated a direct relationship between OD and NGF concentration; thus the higher the OD, the higher the NGF concentration in the sample. The unknown concentrations of NGF in the samples were determined by comparing OD values of the samples to the standard curve (Fig. 1). All calculated linear curves showed a regression coefficient of 0.2099.

Statistical analysis

Analysis of variance was based on a linear model including the fixed effects of sex (males and females), group (healthy, acute and chronic), age, and site of synovial fluid collection (shoulder, elbow and stifle). Least squares means for NGF concentrations for each group were obtained from model solutions. Differences between means for healthy and acute groups, and between means for healthy and chronic groups were tested with an F-test. All analyses were performed using the general linear SAS model procedure⁴. All values were expressed as mean ± SE. Significance level was set at p < 0.05.

Results

Patient data

Group A (Control group) was composed of 12 dogs of various breeds, of which five were male (4 neutered, 1 intact) and seven were female (3 spayed, 4 intact). The mean age and weight were 1.1 years (range: 7 months to 2 years) and 28.5 kg (range: 20 to 50 kg), respectively. Breeds represented were Boxer (n = 3), German Shepherd (n = 2), Dobermann (n = 2), mixed breed (n = 3), Rough Collie (n = 1), and Cane Corso (n = 1). Group B consisted of 16 dogs, of which seven were male (5 neutered, 5 intact) and 12 were female (8 spayed, 4 intact), with a mean age and weight of 4.9 years (range: 9 months to 11 years) and 26.9 kg (range: 7 to 44 kg), respectively. This group included patients with fragmented medial coronoid process (n = 1), ununited anconeal process of the elbow (n = 2), medial patellar luxation (n = 2), and cranial cruciate ligament rupture of the stifle joint (n = 9). The Group C consisted of 22 dogs of which 10 were male (5 neutered, 5 intact) and 12 were female (8 spayed, 4 intact), with a mean age and weight of 4.9 years (range: 9 months to 11 years) and 26.9 kg (range: 7 to 44 kg), respectively. This group included patients with fragmented medial coronoid process (n = 1), ununited anconeal process of the elbow (n = 2), medial patellar luxation (n = 4), cranial cruciate ligament rupture (n = 13), osteochondritis dissecans (n = 1) and synovitis of the stifle joint (n = 1). All details of the 50 dogs enrolled in this study are recorded in the Appendix Table 1 (available online at www.vcot-online.com).

Nerve growth factor concentrations in synovial fluid samples

As shown in the Appendix Table 1 (available online at www.vcot-online.com), the NGF concentration in the synovial fluid of...
healthy dogs (Group A) ranged from 0.60 to 8.40 pg/ml (mean: 3.65 ± 2.18 pg/ml). In Group B, the NGF synovial concentration ranged from 0.60 to 9.50 pg/ml (mean: 6.45 ± 2.45 pg/ml). Comparison between A and B Groups did not reveal any significant difference for NGF synovial values (p = 0.79) (Fig. 2). Conversely, the NGF concentration in the synovial fluid of dogs in Group C ranged from 2.35 to 81.60 pg/ml, with a mean value of 20.19 ± 17.51 pg/ml, and was significantly higher (p < 0.01) compared to Group A values (Fig. 2). No significant effect of sex, age and site of synovial fluid collection was detected (p = 0.245).

**Discussion**

The purpose of this report was to verify the presence of NGF in the synovial fluid of dogs and to detect if there were identifiable differences between healthy dogs and dogs with secondary OA resulting from a variety of joint disorders. To the authors’ knowledge, this is the first quantification of NGF concentrations in normal canine synovial fluid by an ELISA technique specific for this neurotrophin, with an optimal sensitivity and no significant cross-reactivity with other neurotrophins. The concentrations were similar, although lower, than those in human healthy volunteers and healthy athletic horses (Isola M, unpublished data), but differed from the results of Aloe and co-workers who could not detect NGF in normal synovial fluid (22, 27). The use of different NGF assay methods and the masking effect of NGF binding proteins might account for these discrepancies. The present study also showed that the average concentration of NGF in the synovial fluid of dogs with chronic OA lameness was significantly higher than in control dogs and dogs with acute lameness. The increased synovial fluid concentrations of NGF in the inflammatory and non-inflammatory arthritides has been previously described both in humans and in animal models, however the results appear to be inconsistent (22, 27, 28).

Some authors have found increased concentrations of NGF in synovial fluid and sera from patients suffering from rheumatoid arthritis, spondyloarthropathies, psoriatic arthritis, and to a lesser extent, OA (22, 27, 29, 30). Instead, Rihl et al. reported that NGF concentrations were not significantly changed in the synovial fluid of OA patients as compared with the control group (31). The contributory role of NGF in rheumatic diseases, including OA, has been studied extensively (20). Indeed, both in humans and dogs the hypothesis that NGF might somehow be involved in the pathogenic cascade of OA joint inflammation and pain has been supported by several findings such as the NGF receptor expression up-regulation in the synovial compartment, the increased local release of NGF according to the degree of joint inflammation and cartilage degeneration, and the increased synthesis of pro-inflammatory cytokines, such as interleukins 1 and 6 and TNF, directly stimulating the synthesis of NGF (24, 29, 30, 32-35).

**Fig. 2** Concentrations of nerve growth factor in canine synovial fluid from normal joints and joints with acute and chronic secondary osteoarthritis. Box and whisker plots represent the distribution of numerical data within the A (normal), B (acute) and C (chronic) Groups. The bottom and top of the boxplots are the 25th and 75th percentile (the lower and upper quartiles, respectively), and the median is represented by a black line within the boxplot for each of the three groups. Asterisks within the boxplots indicate the mean values of nerve growth factor synovial concentration in all three groups. Error bars represent the minimum and maximum concentrations of nerve growth factor measured in the synovial fluid of normal, acute and chronic OA dogs. NGF = nerve growth factor.
might also suggest that NGF may not be directly responsible for pain but that it is only an epiphenomenon of OA. In our opinion, these findings should be interpreted in relation to some intrinsic limitations to this study, mainly stemming from the high inter- and intra-group variability of the studied sample population, focusing in particular on demographic features such as age and the articular sites selected for the synovial fluid collection. Nevertheless, a preliminary statistical analysis did not reveal a significant interaction of parameters such as age, sex and site of synovial fluid collection on the NGF synovial concentrations between healthy and diseased dogs. Additionally, in the Group C dogs (chronic lameness), the OA varied in location and radiographic severity. Ideally, sub-groups could have been made according to these variables, but this would have lowered the number of dogs and dramatically lowered the power of the statistical evaluation. Another limitation was the inclusion of dogs affected with cranial cruciate ligament rupture in both diseased groups. The acute onset of the disease is very unusual, especially in the medium and large breeds of dogs. This drawback was related to our case selection, resulting in the inclusion of client-owned animals that were not standardized for the degree of gait alterations, and to the assumption that the radiographic degree of OA stifle does not correlate with functional disability of the limb (42). However, despite these limitations, all of which might be possible sources of bias in the evaluation of the results, the data reported here show for the first time that NGF is present in canine synovial fluid and its concentrations are significantly increased in the synovial fluid of dogs with chronic lameness as compared to healthy dogs and dogs with acute lameness. Further studies are needed to identify both the NGF and its receptor system expression within canine articular tissues, and the possible correlation between systemic and synovial NGF concentrations so as to elucidate the role of NGF in the pathophysiology of canine OA inflammation and pain.

Conflict of interest
None declared.

References
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