**Short Communication**

# Nerve growth factor in dogs: Assessment of two immunoassays and selected ocular parameters following a nicergoline challenge per os

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**Abstract**

Impairment of corneal nerves can result in the development of ocular surface diseases such as aqueous tear deficiency and neurotrophic keratopathy. This study investigates oral nicergoline, an α-adrenoceptor antagonist shown to enhance endogenous secretion of nerve growth factor (NGF) by the lacrimal gland, as a potential therapy for these conditions. Five female spayed Beagle dogs received a 2-week course of oral nicergoline (10 mg twice daily). Drug safety was evaluated with ophthalmic and physical examinations, blood pressure monitoring, bloodwork and urinalysis. The effect of nicergoline on the ocular surface was assessed with corneal esthesiometry, Schirmer tear test-1 and tear film breakup time. Drug effect on NGF levels was assessed by collecting tears and blood at baseline and completion of therapy using a bead-based immunoassay and an enzyme-linked immunosorbent assay. Although nicergoline was well tolerated in all dogs, it did not have a significant impact on corneal sensitivity, tear production or tear stability. Of note, NGF was below the limit of quantification in all tear samples, and was only detected in 8/20 serum samples with no significant difference between levels at baseline (189.4 ± 145.1 pg/ml) and completion of therapy (149.4 ± 79.4 pg/ml). Further validation of NGF analytical assays is warranted before nicergoline is investigated in clinical patients.

**Keywords:** Canine, Cochet-Bonnet, ELISA, Neurotrophic keratopathy, Nerve growth factor, Nicergoline

## Introduction

Corneal nerves play a key role in maintaining the anatomical integrity and function of the ocular surface. They provide sensation for blinking and protection of the eye, mediate tear secretion, and supply epithelial and stromal cells with trophic factors important for corneal health.[1,2] Impairment of corneal nerves is common in dogs, whether secondary to diseases such as diabetes mellitus and canine herpesvirus-1,[3,4] or surgical procedures such as transscleral cyclophotocoagulation and ocular evisceration.[5-7] Regardless of the underlying cause, corneal nerve injury can lead to neurotrophic keratopathy, a serious ocular disease characterized by persistent epithelial defects, reduced tear production and impaired wound healing.[2,8] Corneal nerves take months to years to fully regenerate,[2] and although therapeutic intervention can accelerate healing and restore the integrity of the ocular surface, no single therapy has proven effective in a reliable and cost-effective manner to date. Nicergoline, an α-adrenoceptor antagonist described for cognitive disorders such as dementia,[9] enhances endogenous secretion of nerve growth factor (NGF) by the lacrimal gland and could thereby provide sustained levels of NGF in the tear fluid of affected patients. [10,11] Studies on NGF have shown promising therapeutic outcomes in human patients with neurotrophic keratopathy or corneal wounds,[11-15] as well as animal models with herpetic keratitis or dry eye; [16,17] however, topical NGF requires frequent administration to be effective, and is likely cost-prohibitive to most pet owners given the need to compound the drug at clinically relevant concentrations. The present study investigates the safety and usefulness of oral nicergoline as a potential alternative to topical NGF therapy in dogs. Following a nicergoline challenge per os, various systemic and ocular parameters were monitored and NGF levels in tears and plasma were assessed using two different immunoassays.

**Material and Methods**

Five female spayed Beagle dogs (1.5-2.0 years, 7.5-10.0 kg) were enrolled in the study and confirmed to be healthy based on physical and ophthalmic examinations. The sample size was based on *a priori* power calculation aimed at detecting a 50% change in lacrimal NGF levels between baseline and post-nicergoline therapy,[11] assuming a power of 80% and a significance level of 0.05. The study was approved by the Institutional Animal Care and Use Committee of Iowa State University. Each dog received 10 mg of nicergoline [Сермион® (Sermion), Pfizer Inc., Russia] orally twice daily for 2 consecutive weeks. Of note, the drug is no longer produced by the manufacturer and was therefore obtained through an online pharmacy (nootropicspot.com) for the present study. Physical and ophthalmic examinations were performed daily throughout nicergoline administration. In addition, the following procedures were performed at baseline and at completion of the 2-week course: (*i*) complete blood count (CBC), serum biochemistry, and urinalysis to assess for systemic toxicity; (*ii*) indirect blood pressure monitoring (Doppler model 811-B, Parks Medical Electronics, Las Vegas, NV) due to the vasodilatory properties of nicergoline;[9] (*iii*) corneal esthesiometry (Cochet-Bonnet aesthesiometer, Luneau Ophthalmologie, Chartres, France), Schirmer tear test-1 (STT-1; Eye Care Product Manufacturing, LLC, Tucson, AZ, USA) and tear film breakup time (TFBUT; Ful-Glo®, Akorn Inc., Buffalo Grove, IL, USA) to assess for drug effect on the ocular surface with each diagnostic test performed 10 min apart;[18] and (*iv*) blood and tear collection to assess for drug effect on NGF levels, obtained prior to and 1h following the morning drug administration (Day 0 and Day 14). Blood was collected by jugular venipuncture, transferred into plain tubes, centrifuged at 1232g for 10 minutes, and the harvested serum was stored in 2-mL cryovials at -80°C. Tear fluid was collected in each eye with a Schirmer strip placed in the ventrolateral conjunctival fornix until 20-mm wetness was reached, followed by extraction of tears from the filter paper using one of two methods: (*i*) elution in phosphate-buffered saline (Gibco® PBS, pH 7.2, Thermo Fisher Scientific, Rockford, IL, USA) for samples collected on week 1, *i.e.* Schirmer strips cut into small pieces, placed in 400 µL of PBS for 3h at +4°C, followed by ultrasonic bath for 30min, centrifugation at

3824g for 10min, and storage of the supernatant in 2-mL cryovials at -80°C; or (*ii*) centrifugation for samples collected on week 2, *i.e.* Schirmer strips placed in 0.2-mL vials that were previously punctured at their bottom with an 18-gauge needle, followed by centrifugation into 2-mL tubes at 3,884g for 2 minutes and storage of tear fluid at -80°C. NGF levels were analyzed with either a bead-based immunoassay (NGF canine ProcartaPlex™ Simplex, Invitrogen™, Waltham, MA, USA) for half of the samples (random selection), or an enzyme-linked immunosorbent assay (Human beta-NGF DuoSet ELISA, R&D)[11] for the other set of samples. Statistical analysis was performed using SigmaPlot version 14.0 (Systat Software, Point Richmond, CA), and values $P < 0.05$ were considered statistically significant.

**Results**

Nicergoline was well tolerated in all dogs, with no noticeable changes in physical and ophthalmic examinations at any observable time point. Oral dosing with nicergoline did not affect systolic blood pressure, with measures obtained at baseline (128.1 ± 24.4 mmHg) being comparable to those at the end of nicergoline therapy (124.5 ± 13.0 mmHg; paired t-test, $P = 0.637$). Additionally, results from the complete blood count, serum biochemistry and urinalysis did not differ significantly between baseline and completion of therapy for all parameters (paired t-tests, $P \geq 0.057$, **Table 1**), except for a statistically (but not clinically) significant decrease in total protein levels (5.94 ± 0.24 gm/dl *vs.* 5.7 ± 0.27 gm/dl, respectively; $P < 0.001$) and total calcium levels (10.70 ± 0.29 mg/dl *vs.* 10.56 ± 0.23 mg/dl, respectively; $P = 0.025$). No differences were noted between the right and left eyes for any ocular diagnostic test (paired *t*-tests, $P \geq 0.637$), hence the average measurements from both eyes was used for statistical analysis. Mean (± standard deviation) values did not differ significantly between measurements obtained at baseline *vs.* completion of nicergoline therapy (paired *t*-tests, $P \geq 0.091$) for corneal tactile sensation (2.4 ± 0.3 *vs.* 2.3 ± 0.3 cm, respectively, **Figure 1A**), STT-1 (17.4 ± 2.6 *vs.* 19.1 ± 0.9 mm/min, respectively, **Figure 1B**), and TFBUT (14.0 ± 3.4 vs. 17.3 ± 4.0 seconds, respectively, **Figure 1C**).

Internal standards for NGF provided accurate standard curves for both ProcartaPlex™ assay (5 parameters logistic regression, **Figure 2A**) and ELISA (second-degree polynomial regression, $R^2$ = 0.9996, **Figure 2B**). However, NGF levels were below the lower limit of quantification in all tear samples (3.9 and 31.3 pg/ml for ProcartaPlex™ and ELISA assays, respectively), and were only detectable with ELISA in 2/5 serum samples at baseline (86.7 and 292.0 pg/ml) and 6/15 serum samples collected after nicergoline administration (149.4 ± 79.4 pg/ml, 54.9-269.2 pg/ml). Nicergoline therapy did not significantly increase serum levels of NGF in dogs (paired t-test, $P$ = 0.620).

## Discussion

Nicergoline was well tolerated in dogs, although the drug did not affect the canine ocular parameters in a significant manner. Interestingly, tear production (STT-1) and tear stability (TFBUT) appeared to increase with nicergoline therapy, although the changes were not statistically significant and may be related to measurement noise given the low sample size and lack of control group. The latter represents a limitation of the study, and is important in establishing a causal relationship between drug administration and progression of clinical parameters, especially given the inherent variability of many ocular surface diagnostics in veterinary medicine.[18] The inability to quantify NGF in canine tears is surprising, especially given the following measures undertaken in the present study: relatively high oral dosing (2-2.7 mg/kg/d *vs*. 0.5 mg/kg/d for behavioral disorders),[20] two collection time points (to capture trough and peak concentrations), two different extraction methods (elution in PBS and centrifugation),[21] and two different analytical assays. Similarly, NGF was not detected in tear fluid of dogs using a multiplex bead-based immunoassay,[22] albeit other chemokines/cytokines such as interleukin-8 and interferon-gamma were quantifiable at relatively high concentrations using the same analytical method.[22,23] In contrast, Woo and colleagues detected high levels of NGF in canine tears (15.4 ± 4.6 ng/mL);[24] however, the ELISA test used in that study is no longer commercially available, and the assay guidelines discourage the use of this kit for samples rich in IgG (such as serum and tears) as it can lead to cross-

reactivity with NGF and falsely elevate absorbance readings (personal communication with manufacturer). Taken together, we believe that the apparent absence of lacrimal NGF in our canine subjects is related to inadequate analytical assays, and further tests validations are required before this promising growth factor is investigated in patients with neurotrophic keratopathy. Further, the adsorptive properties of Schirmer strips could theoretically impact NGF quantification, as shown for other proteins,[25] hence other methods for tear collection could be considered in future studies (*e.g.* silicone tubing,[24] microcapillary tubes,[26] or ophthalmic sponges[27-28]).

## Conflict of interest statement

The authors declare no conflicts of interest.


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**Table 1.** Results of complete blood count, serum biochemistry and urinalysis in 5 healthy female spayed Beagle dogs, assessed at baseline and following 2 weeks therapy with oral nicergoline (10 mg twice daily). WBC = white blood cells; RBC = red blood cells; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; RDW = red cell distribution width; MPV = mean platelet volume; BUN = blood urea nitrogen; Alk Phos = alkaline phosphatase; ALT = alanine aminotransferase; N/A = Not applicable. P-values depict results of paired t-tests, and are considered significant is $P < 0.05$ (in bold).

| | Parameter | Reference range | Baseline | Post- therapy | P-value |
|---|---|---|---|---|---|
| **Complete blood count** | WBC ($x10^3$/ul) | 6 – 17 | 6.6 ± 0.9 | 6.6 ± 1.2 | 0.944 |
| | RBC ($x10^3$/ul) | 5.5 – 8.5 | 6.7 ± 0.5 | 6.4 ± 0.4 | 0.126 |
| | Hemoglobin (gm/dl) | 12 – 18 | 15.3 ± 0.9 | 14.6 ± 0.5 | 0.057 |
| | Hematocrit (%) | 37 – 55 | 45.6 ± 2.5 | 42.7 ± 3.0 | 0.061 |
| | MCV (fl) | 60 – 77 | 67.9 ± 2.3 | 68.3 ± 2.1 | 0.062 |
| | MCH (pg) | 19.5 – 30 | 22.8 ± 0.8 | 22.6 ± 0.8 | 0.330 |
| | MCHC (gm/dl) | 32 – 36 | 33.5 ± 0.2 | 33.2 ± 0.7 | 0.214 |
| | RDW (%) | 11.6 – 14.8 | 12.9 ± 0.3 | 13.2 ± 0.3 | 0.178 |
| | Platelet ($x10^3$/ul) | 200 – 500 | 306.6 ± 68.9 | 319.8 ± 54.3 | 0.564 |
| | MPV (fl) | 7 – 11 | 8.0 ± 1.0 | 7.7 ± 0.9 | 0.315 |
| | Neutrophil ($x10^3$/ul) | 3.0 – 11.4 | 4.0 ± 0.9 | 4.3 ± 1.2 | 0.661 |
| | Lymphocyte ($x10^3$/ul) | 1.0 – 4.8 | 2.0 ± 0.4 | 1.8 ± 0.3 | 0.414 |
| | Monocyte ($x10^3$/ul) | 0.15 – 1.35 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.427 |
| | Eosinophil ($x10^3$/ul) | 0 – 0.75 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.893 |
| | Basophil ($x10^3$/ul) | 0 – 0.1 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.871 |
| **Serum biochemistry** | Sodium (mEq/L) | 141 – 151 | 146.4 ± 1.5 | 147.0 ± 0.7 | 0.573 |
| | Potassium (mEq/L) | 3.9 – 5.3 | 4.4 ± 0.3 | 4.1 ± 0.3 | 0.207 |
| | Chloride (mEq/L) | 112 – 121 | 116.4 ± 1.8 | 116.2 ± 0.8 | 0.861 |
| | Bicarbonate (mEq/L) | 19 – 25 | 22.4 ± 1.3 | 22.6 ± 1.1 | 0.847 |
| | Calcium (mg/dl) | 9.7 – 11.3 | 10.7 ± 0.3 | 10.6 ± 0.2 | **0.025** |
| | Phosphorus (mg/dl) | 3.2 – 6.0 | 3.7 ± 0.6 | 4.0 ± 0.3 | 0.092 |
| | Magnesium (mg/dl) | 1.7 – 2.5 | 1.9 ± 0.2 | 1.8 ± 0.2 | 0.275 |
| | BUN (mg/dl) | 10 – 30 | 15.8 ± 4.1 | 15.4 ± 1.8 | 0.803 |
| | Creatinine (mg/dl) | 0.5 – 1.5 | 0.7 ± 0.2 | 0.7 ± 0.2 | 0.704 |
| | Glucose (mg/dl) | 68 – 115 | 92 ± 11 | 92.6 ± 2.7 | 0.922 |
| | Total protein (gm/dl) | 5.2 – 7.1 | 5.94 ± 0.24 | 5.70 ± 0.27 | **< 0.001** |
| | Albumin (gm/dl) | 2.7 – 4.0 | 3.3 ± 0.2 | 3.3 ± 0.2 | 0.553 |
| | Alk Phos (IU/L) | 20 – 150 | 43.6 ± 17.9 | 41.4 ± 17.4 | 0.282 |
| | ALT (IU/L) | 19 – 80 | 35.8 ± 4.1 | 37.8 ± 6.3 | 0.230 |
| | Total bilirubin (mg/dl) | 0.1 – 0.6 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.258 |
| | Cholesterol (mg/dl) | 132 – 300 | 155.8 ± 28.7 | 150.6 ± 24.6 | 0.270 |
| | Triglycerides (mg/dl) | 24 – 115 | 43.4 ± 18.9 | 29.0 ± 4.9 | 0.182 |
| | Anion gap | 8 – 17 | 13 ± 1 | 12.2 ± 1.1 | 0.242 |
| **Urinalysis (cystocentesis)** | Specific gravity | 1.015 – 1.055 | 1.043 ± 0.012 | 1.051 ± 0.004 | 0.221 |
| | Urine pH | 5.5 – 6.5 | 6.3 ± 0.3 | 6.2 ± 0.3 | 0.374 |
| | White cells (/hpf) | < 3 | 0 | 0 | 1.000 |
| | Red cells (/hpf) | < 10 | 0 | 0 | 1.000 |
| | Crystals | 0 | 0 | 0 | 1.000 |
| | Bacteria | 0 | 0 | 0 | 1.000 |
| | Epithelial cells | None to rare | Rare | Rare | N/A |
| | Glucose (mg/dl) | Negative | Negative | Negative | N/A |
| | Bilirubin | Negative to 1+ | Negative | Negative | N/A |
| | Ketones | Negative | Negative | Negative | N/A |
| | Proteins | Negative | Negative | Negative | N/A |

**Figures**

**Figure 1.** Box-and-whisker plots depicting the results of corneal tactile sensation (A), Schirmer tear test-1 (B) and tear film breakup time (C) in 5 healthy female Beagles dogs before (white box) and after (gray box) receiving a 2-week course of oral nicergoline (10 mg twice daily). Each plot represents the mean (dotted line), median (solid line), 2.5$^{th}$ percentile (lower whisker), 25$^{th}$ percentile (lower limit of box), 75$^{th}$ percentile (upper limit of box), and 97.5$^{th}$ percentile (upper whisker).

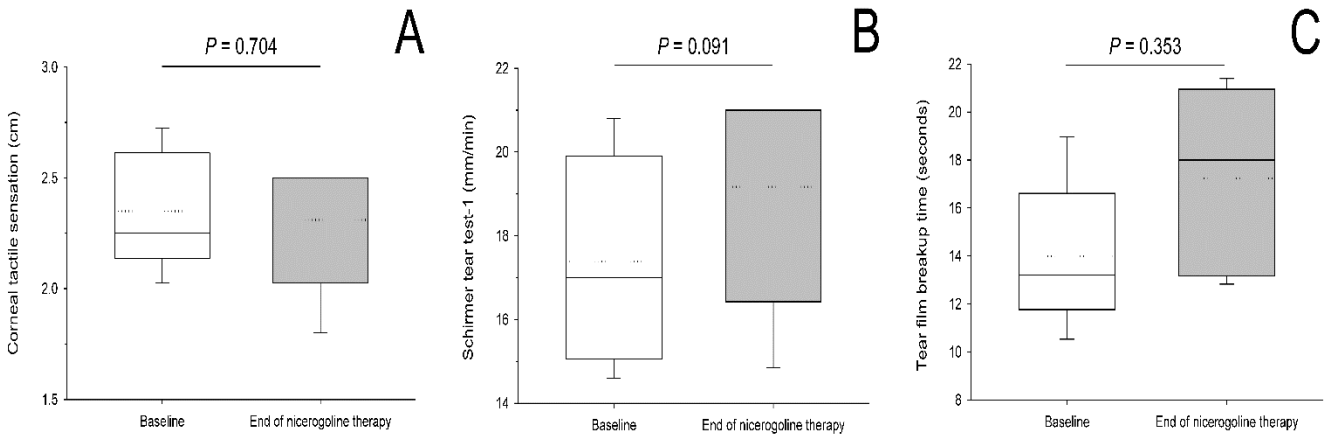

**Figure 2.** Standard curves (in blue) obtained with internal standards of nerve growth factor (NGF) with either bead-based immunoassay (A) or an enzyme-linked immunosorbent assay (B).