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ANXITANE® tablets reduce fear of human beings in a laboratory model of anxiety-related behavior

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Abstract Anxiety and fear are common underlying factors in many canine behavior problems that impair the human–pet bond and often result in abandonment, relinquishment, or euthanasia. A combination of behavioral and pharmacological interventions is used to ameliorate the behavioral signs associated with anxiety-related behaviors in dogs, but there continues to be need for effective interventions. The current study examined the effects of the nutraceutical ANXITANE® (L-Theanine) chewable tablets on fear of unfamiliar human beings. We first characterized dogs as anxious on the basis of the existence of a fear response to human beings in their home-pen. We then demonstrated that dogs characterized as anxious (N = 10) showed reduced interaction with an unknown human being as compared with normal controls (N = 7). The effect of an administration of ANXITANE® tablets (N = 5) on these anxious Beagle dogs was compared with placebo (N = 5). Objective behavioral measures of anxiety were obtained using an open-field test, a human interaction test, and an actiwatch protocol that allowed monitoring of activity over 24-hours. The ANXITANE® tablets-treated dogs showed greater human interaction and approach than the placebo control group, and no side effects related to treatment, including motor stimulant or sedative effects, were seen. The current study suggests that ANXITANE® tablets are effective for reducing fearful behavior toward unfamiliar human beings in dogs and supports their use for treating anxiety-related behaviors.
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Introduction

Fear and anxiety are underlying factors in many behavior problems for which animals are referred to veterinary behaviorists. Examples include separation anxiety, noise phobias (e.g., thunderstorms), housesoiling in response to

an anxiogenic stimulus, and many forms of aggression (Denenberg et al., 2005). In addition to the physical damage that the pet may cause to the household, itself, or others, fear and anxiety also lead to physiological changes that may affect the pet's health, including stimulation of the hypothalamic-pituitary-adrenal axis and increased release of noradrenalin, adrenaline and, in chronic states of anxiety, prolactin (Clark et al., 1997; Hennessey et al., 1998; 2001; Dreschel and Granger, 2005; Frank et al., 2006; Pageat et al., 2007, Beata and Schwobthaler, 2009). Chronic stress,

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which can result from untreated or ongoing anxiety, can contribute to a wide range of medical conditions including gastrointestinal, dermatologic, and urinary tract disorders, as well as immunosuppression (Siebert and Landsberg, 2008). Thus, effective treatment of anxiety disorders is not only a necessity for the health and welfare of the pet, but also to improve the problem for the owner and ultimately restore the bond between owner and pet. This, in turn, can help reduce abandonment or euthanasia of the pet (Miller et al., 1996; Lue et al., 2008). In fact, behavior problems are the most common cause of canine relinquishment (Houpt and Reisner, 1996; Miller et al., 1996; Gorodetsky, 1997).

Both clomipramine (a tricyclic antidepressant) and fluoxetine (a serotonin reuptake inhibitor) are licensed in the United States for the treatment of separation anxiety in dogs as an adjunct to a program of behavior modification (Sherman and Mills, 2008). These drugs may take a month or longer to achieve their full therapeutic effect limiting their short-term utility, although clinical effects might begin to be seen in as early as 1 week (Frank et al, 2006; Simpson, 1997). Side effects may include gastrointestinal signs such as decreased appetite. At high doses, or when used in combination with other drugs that enhance serotonin transmission, the pet can develop a serious and potentially fatal condition known as serotonin syndrome, which includes signs of confusion, agitation, hyperthermia, tachycardia, nausea, diarrhea, muscle tremors, and coma. Therefore, these drugs should not be used concurrently with monoamine oxidase inhibitors, such as amitraz and selegiline, or with other antidepressants (Crowell-Davis and Murray, 2006). Although not licensed for the treatment of anxiety disorders in dogs, benzodiazepines are commonly used for their short-term or immediate effects, such as before veterinary visits or as adjunctive therapy in the treatment of separation anxiety or storm phobias (Herron et al., 2008, Sherman and Mills, 2008). However, the duration of effect, dose, and therapeutic efficacy is not well established in dogs and the potential side effects, including ataxia, sedation, paradoxical excitation, disinhibition (leading to an increased possibility of aggression), and a rebound effect on withdrawal (Plumb, 2005, pp. 238), often result in treatment discontinuation (Herron et al., 2008). Numerous natural products are also marketed for the treatment of anxiety in dogs; however, few, if any, of these products have any documented evidence of efficacy in veterinary behavior with the exceptions of DAP® (Ceva Animal Health, Manchester, MO), a pheromone product that has a calming effect on some dogs (Levine et al., 2007; Mills et al., 2006; Tod et al., 2005), and, alpha-casozepine, (Schering-Plough, Middlesex, UK), a bovine milk protein that did not differ in the reduction of EDED (evaluation of dog's emotional disorder) when compared with selegiline (Beata et al., 2007). The exceptions notwithstanding, it is clear that there is a limited availability of therapeutics for canine anxiety disorders and a there is continued need for novel safe and effective products.

ANXITANE® tablets (Virbac Animal Health, Fort Worth, TX) are a nutraceutical containing 99.95% pure L-theanine (N-ethyl-L-glutamine), an amino acid found largely in green tea. L-theanine may increase concentrations of gamma amino-butyric acid, an inhibitory neurotransmitter, and increase brain serotonin and dopamine (Nathan et al., 2006). ANXITANE® tablets are palatable and showed no side effects when given at 5 times the recommended dose (http://www.virbacvet.com/images/resources/other/anxitane_firstintention.pdf). In a preliminary study of 12 dogs with noise phobias, dogs treated with ANXITANE® tablets plus behavior modification showed greater behavioral improvement than dogs treated with behavior modification alone. The differences, however, did not reach statistical significance (Berteselli and Michelazzi, 2007). In a nonplacebo controlled open-label clinical trial, there was a significant reduction in global anxiety scores, including a reduction in fear of people (in the street) after a 2-month treatment with ANXITANE® tablets (Kern, 2005, pp. 191-196), suggesting ANXITANE® tablets are effective for treating anxiety-related behaviors.

The primary objective of this study was to obtain data on the efficacy of ANXITANE® tablets compared with placebo control on an anxiety-related behavior in a laboratory model; more specifically, the fear of unknown human beings. We used an open-field and a human-interaction test to objectively assess the effects of ANXITANE® tablets on fear of human beings and behavioral activity (Siwak et al., 2001). To identify fearful dogs, we developed a novel assessment on the basis of the occurrence of a fear response to human beings in their home-pens and on interaction duration with an unknown human being in the human-interaction test. We previously reported that dogs that show a fear response to human beings in their home-pen also show reduced interactions with human beings in the human-interaction test (Landsberg et al., 2009). In the current study, we hypothesized that treatment with ANXITANE® tablets would reduce the fear response to human beings, which would be evident by increased interaction with and approach to the human being in the human-interaction test.

Materials and methods

Subjects

A total of 21 healthy Beagle dogs aged 2.6–7.0 years and weighing 7–17 kg were initially tested at baseline. Seven of the animals served as normal control. The remaining 14 animals initially were considered to show fear of human beings based on their response when approached in their home-pens (Landsberg, et al., 2009); these dogs were initially identified as fearful or anxious by a veterinary behaviorist (G.M.L.) and ECVBM-CA resident (S.D.) on the basis of facial expression, body postures, and actions including retreat, hiding, and conflict behaviors such as circling. By comparison, nonfearful or nonanxious dogs were those that

approached and remained at the front of their home-pens and showed greeting behaviors to strangers. After baseline testing, four anxious dogs that interacted with the unknown human being for more than 90 seconds were excluded. The remaining 10 fearful dogs were randomly divided into two groups of five subjects and assigned either to treatment or placebo. After 8 weeks, these dogs were re-assessed to determine the effects of the treatment. Dogs were group-housed, were fed once daily with a suitable maintenance diet, and provided with water ad libitum.

General design

Before the commencement of the treatment phase, baseline testing was conducted using the open-field and human-interaction tests. In addition, a measure of 24-hour day/night activity was obtained. The test phase consisted of a blinded laboratory efficacy study with one placebo and one treatment (ANXITANE® tablets) group, with five fearful dogs per group. Specifically, technicians collecting behavioral data were blind to treatment condition. A random assignment procedure was used to place dogs into groups. The animals in each group were handled and cared for identically throughout the study and all procedures were conducted in accordance with the guidelines set forth by the Canadian Council for Animal Care. The treatment phase occurred over an 8-week interval, with the animals retested on baseline measures during the last week of treatment. To determine whether any side effects related to treatment were evident, trained technicians observed the subjects twice daily for the duration of the study. Any abnormal findings were reported to the veterinarian, who determined whether the findings were related to treatment.

Administration of supplement

One half tablet (50 or 100 mg tablet of ANXITANE® tablets or identically sized placebo) was administered to each subject once in the morning and once in the afternoon for the duration of the treatment period. Both ANXITANE® tablets and placebo were provided by Virbac Animal Health (Fort Worth, TX), were identical in appearance, and were administered identically. Dogs in the treatment group weighing less than 10 kg received 25 mg of ANXITANE® tablets twice daily; those weighing more than 10 kg received 50 mg of ANXITANE® tablets twice daily. A technician not involved in behavioral testing administered the tablet and observed the dogs for 0.5 hours to ensure there was no voiding of the tablet or cross contamination. Weekly body weights were used to determine doses for the subsequent week and treatment was administered for 57 days.

Behavioral measures

The open-field and human-interaction tests have been described previously (Siwak et al., 2001). Briefly, the open-field test is used to objectively score exploratory behavior in an empty open-field arena and provides objective measures of distance traveled, inactivity, and general behavior. The human-interaction test is a variant of this task in which a human being is placed in the center of the open-field arena and instructed not to interact with the dog. Interactions with the human beings are then scored objectively to provide measures of interaction and time spent with human beings.

In the current study, the open-field arena consisted of a room approximately 2.74 × 3.66 m. The open-field arena was sanitized with 1.5% Spray Nine® (Spray Nine Corporation, Johnstown, NY) diluted with water before each test to reduce olfactory cues from the previous test subject. The dog was placed into the arena for 10 minutes per test and the test session was recorded digitally. Behavioral measures were recorded using Ethovision v.3 analysis software (Noldus Information Technology Inc, Leesburg, VA) which tracks the path of the dog to provide a measure of distance and allows the observer to score other defined behaviors. In addition to distance traveled, a trained observer scored both the duration and frequency of inactivity bouts. For the human-interaction tests, frequency and duration of interaction with, and frequency and duration of approach to, an unfamiliar human being were also measured. Interaction with the human being was defined as touching or sniffing the human being and human approach was defined as any part of the animal being within an 80 cm radius of the human being.

Twenty-four hour activity levels were obtained with the use of the Actiwatch system (Mini Mitter, Bend, OR), which is an accelerometer that can be placed on an individual animal and it continuously records movement over extended periods and has been described previously (Siwak et al., 2003). The recorders were placed into a protective casing that was fastened to a cloth collar attached to the neck of each animal at the beginning of the study. At the end of data collection, the recorder data were downloaded to a computer by a dedicated system, which then permitted total day and total night activity counts to be calculated. The recorders were set to continuously record at an epoch length of 0.5 seconds. The Actiwatch data used in the study was obtained on days on which no behavioral testing occurred, to avoid any confounds of the tests on normal patterns of activity. Specifically, the mean day and night activity counts over three consecutive week days immediately after baseline open-field testing and immediately before the treatment time-point open-field testing was used.

The Mann–Whitney U test was used for statistical comparisons between the normal and fearful dogs at baseline. For the comparison between placebo and treatment in fearful dogs, the baseline data was analyzed first using the Mann–Whitney U test. If baseline differences were not present on a given measure, then the treatment-time-point data were analyzed identically. If baseline differences were found, a difference score was calculated and subsequently analyzed.
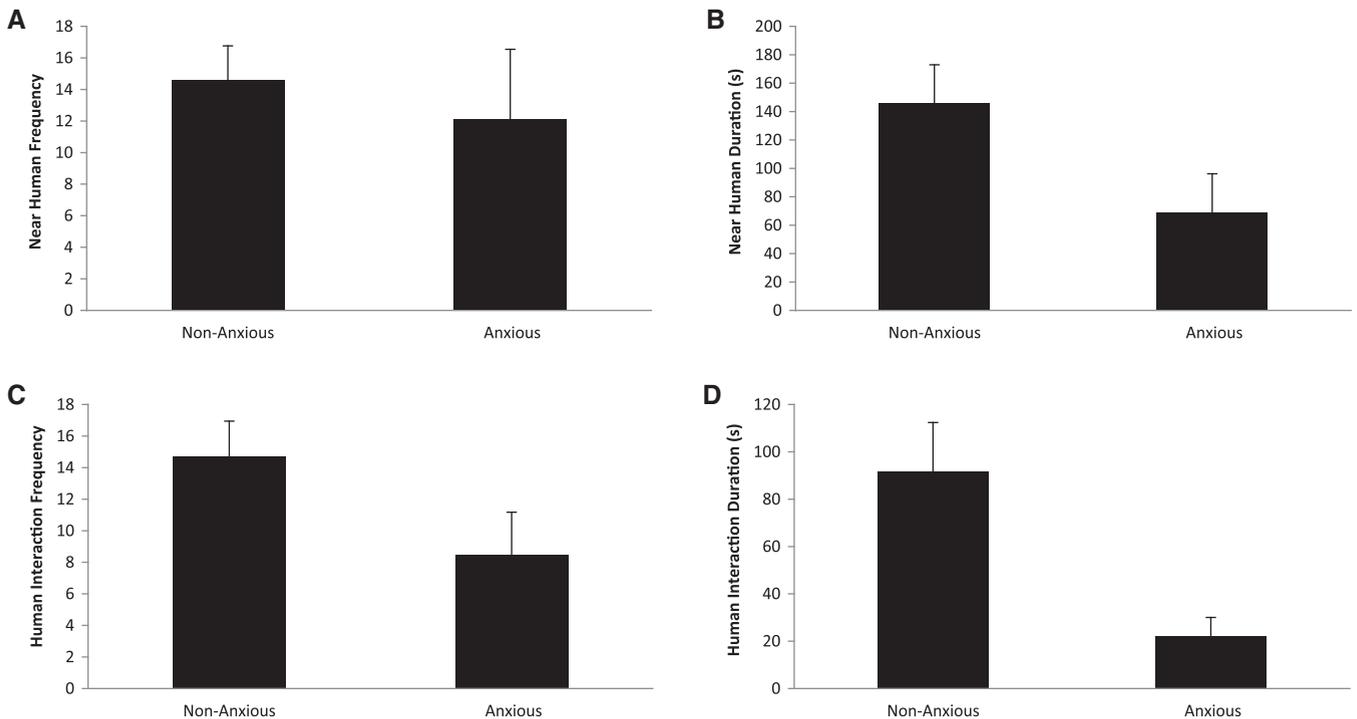
Figure 1   Four baseline measures on the human-interaction test showing mean + SEM of anxious (N = 10) and normal (N = 7) dogs. (A) No differences were found in the mean frequency of approaches to the unknown human being. An approach was considered being within 0.5 m of the human being. (B) Mean duration of time spent near the human being was marginally lower in anxious dogs compared with normals. (C) Mean frequency of interaction with the unknown human being was also marginally lower in anxious dogs compared with normals. (D) Mean duration of human interaction was significantly reduced in anxious dogs compared with normals. Error bars represent SEM.

The Statistica 6.0 (Statsoft Inc., Tulsa, OK) statistical analysis package was used to analyze all data with statistical significance set to $P = 0.05$. If the values were $P \leq 0.1$, the results were deemed to be marginally significant.

Results

For baseline testing, each measure was analyzed using the Mann–Whitney U test with subpopulation (fearful vs. non-fearful) serving as the independent variable. Time spent interacting with the human beings (Figure 1) was significantly lower in the fearful animals (U = 5, $n_1 = 10$, $n_2 = 7$, $P = 0.0034$) compared with normals. Non-significant differences included reductions in both time spent near the human beings (U = 15, $n_1 = 10$, $n_2 = 7$, $P = 0.0510$) and frequency of human interactions (U = 18, $n_1 = 10$, $n_2 = 7$, $P = 0.0971$) in fearful dogs compared with normals. No differences were found between the groups on the open-field measures.

After the fearful dogs were randomly assigned into treatment groups for inclusion in the treatment phase of the study, the Mann–Whitney U test was used to compare the two groups (ANXITANE® tablets vs. placebo) on the human-interaction, open-field, and Actiwatch tests. No baseline differences were found on the human interaction or open-field tests (Figures 2, 3). The results of the human-interaction test revealed significantly longer time

spent near (U = 3, $n_1 = n_2 = 5$, $P = 0.0472$) and interacting (U = 2, $n_1 = n_2 = 5$, $P = 0.0283$) with the human being, and a significant increase in interaction frequency (U = 2, $n_1 = n_2 = 5$, $P = 0.0283$) in the treated group compared with placebo.

No effects of ANXITANE® tablets were detected on the open-field measures (Figure 3). For 24 hour activity rhythms, baseline differences were found because of higher activity counts in the treatment group (Figure 4). Consequently, difference scores for the mean treatment time-point from baseline were analyzed with the Mann–Whitney U test; no treatment differences were found on either day or night mean activity counts. Finally, no adverse events related to treatment occurred over the course of the study.

Discussion

In this study, fearful laboratory Beagle dogs were selected from a larger population and used to examine the anxiolytic effects of ANXITANE® tablets. We used both the open-field and human-interaction tests to objectively assess behavioral measures. The main findings were that the fearful dogs showed reduced interaction with an unknown human being compared with normal dogs and that ANXITANE® tablets improved measures of human approach and interaction compared with placebo.
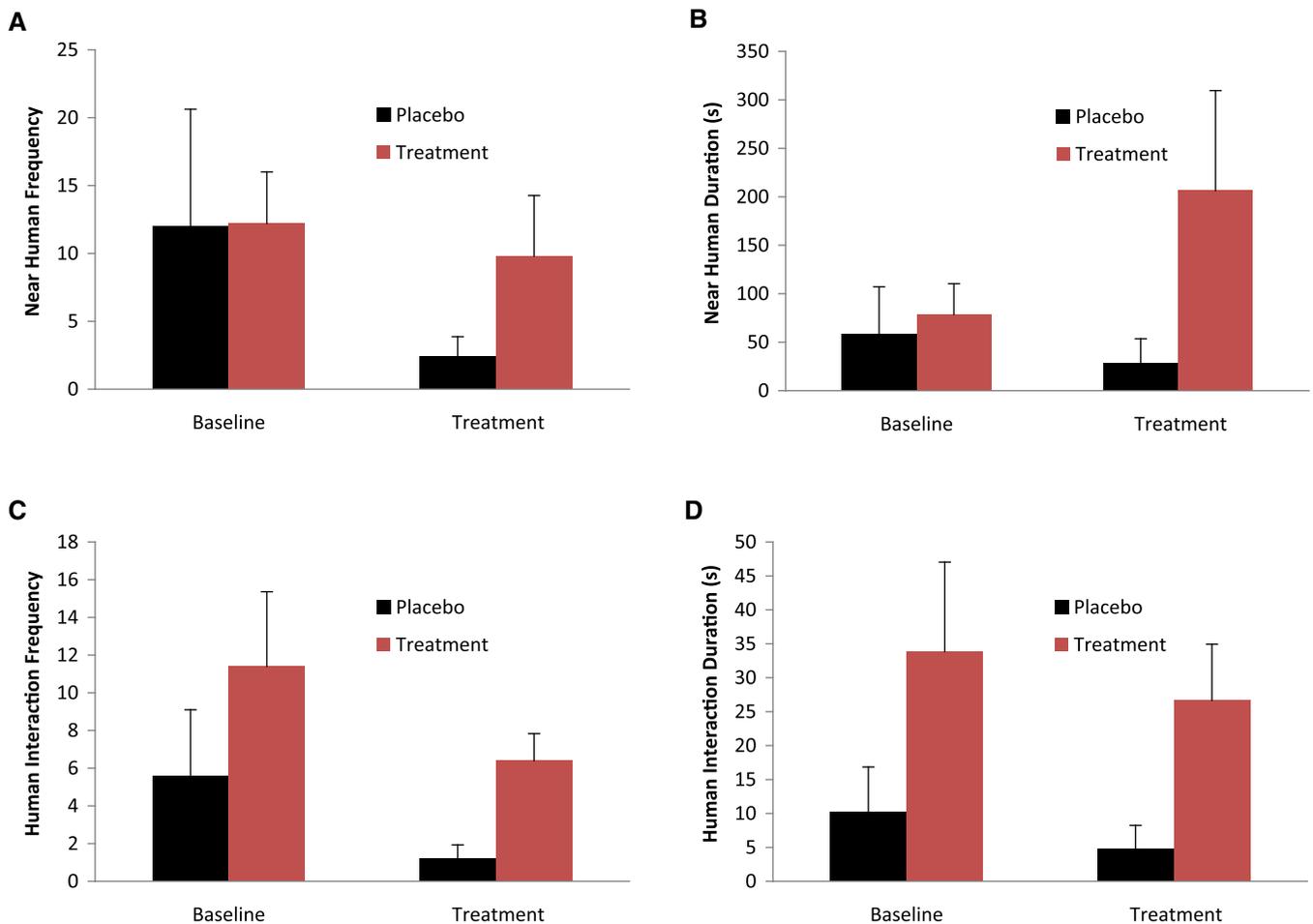
Figure 2 Four baseline and treatment time-point measures for anxious dogs on the human-interaction test showing mean + SEM of placebo (N = 5) and treatment (N = 5) dogs. No differences were found between groups at baseline. (A) No differences were found in the mean frequency of approaches to the unknown human being either at baseline or at treatment. (B) Mean duration of time spent near the human being was significantly higher in treatment dogs at the treatment time-point compared with placebo, likely due to increases over baseline in the treatment group and decreases from baseline in the placebo group. (C) Mean frequency of interaction with the unknown human being was also significantly higher in the treatment group compared with placebo at treatment, although both groups showed mean reductions compared with baseline (albeit to a greater extent in the placebo group). (D) Mean duration of human interaction was significantly higher in treatment dogs compared with placebo, but both groups showed mean reductions from baseline with the largest decreases in the placebo group.

The characterization of fearful vs. nonfearful subjects was initially based on the observation that most laboratory dogs approached the front of their home-pen when a human being entered the housing room. By contrast, a small number of dogs show a persistent fear response to human beings who approached their pen, including escape (moving to the back of the pen), hiding in or behind their home cage enclosures, and displacement (circling) behaviors, all of which were verified by a veterinary behaviorist. We previously demonstrated that these fearful dogs showed reduced human interaction and locomotor activity compared with normal dogs on the human-interaction and open-field tests, respectively (Landsberg et al., 2009). In the current study, an additional exclusion criterion for fearful dogs was used in which dogs that interacted with the human beings for a minimum of 90 seconds (or 15%) of the test

were excluded. This additional exclusion was required because some of the subjects that show a fear response in their home-pen showed an adaptation to the presence of a human being in the human-interaction test with repeated testing. In the current study, four of 14 (28.6 %) dogs were excluded for this reason.

To confirm that the human-interaction test was sufficiently sensitive, we compared this group of fearful dogs to those that showed a normal response in the home-pen. Similar to our previous findings (Landsberg et al., 2009), fearful animals, compared with normals, showed a significant reduction in interaction duration with as well as a marginally significant reduction in time spent near the human beings (reduced human approach) and in interaction frequency with the human beings. Unlike the previous study, differences in total distance traveled were not found in
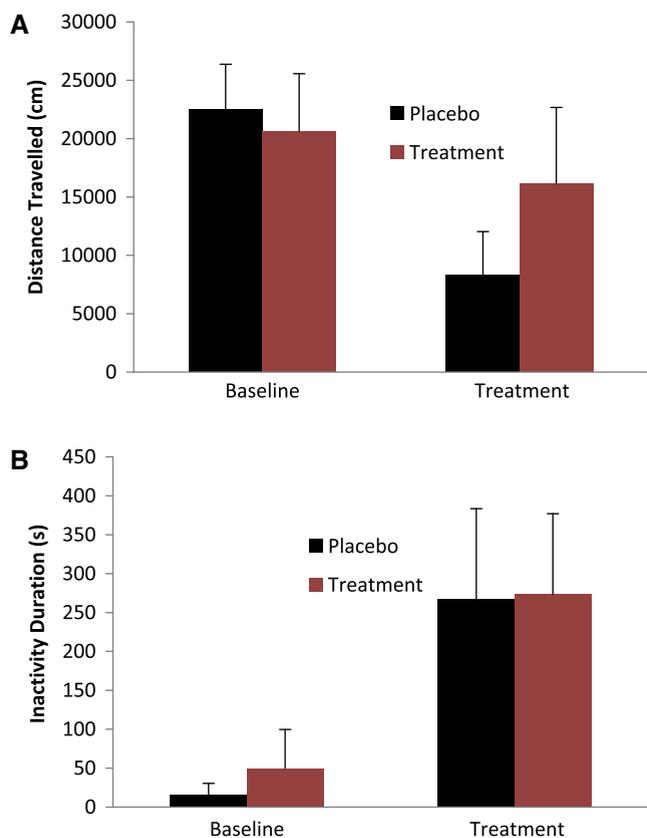
Figure 3 Two baseline and treatment time-point measures, distance traveled in cm (A) and inactivity duration in seconds (B) for anxious dogs on the open-field test showing mean + SEM of placebo (N = 5) and treatment (N = 5) dogs. No differences were found between groups at baseline or at treatment.

Figure 4 Mean day (A) and night (B) activity counts over three consecutive week days for anxious dogs showing mean + SEM of placebo (N = 5) and treatment (N = 4) dogs. Baseline differences were found and consequently difference scores were analyzed. No treatment effects were found.

fearful dogs compared with normals, suggesting that measures related to human interaction and human approach are more robust measures using these subjects.

The use of objective markers, in contrast to subjective markers, was essential in allowing us to determine the effects of ANXITANE® tablets on fearful dogs even though the group sizes (N = 5) were small. Our laboratory originally developed open-field tests to distinguish the effects of age on general behavior in a controlled environment (Siwak et al., 2001) and to examine the effects of drugs on behavior. For example, adrafanil and selegiline increase locomotor activity and stereotypical behavior, respectively (Siwak et al., 2000; Milgram et al., 1993). In the current study, we used two of these tests to assess the effects of ANXITANE® tablets. The first was the standard open-field and the second was the human-interaction test. Both were identical to tests that have been previously described (Siwak et al., 2001), except that a human being unfamiliar to the dog was used in this study with the rationale that this would exacerbate fearful behavior. On the human-interaction test at the treatment time-point, frequency and duration of interaction as well as time spent near the human being was increased by ANXITANE® tablets compared to placebo, which supports an anxiolytic
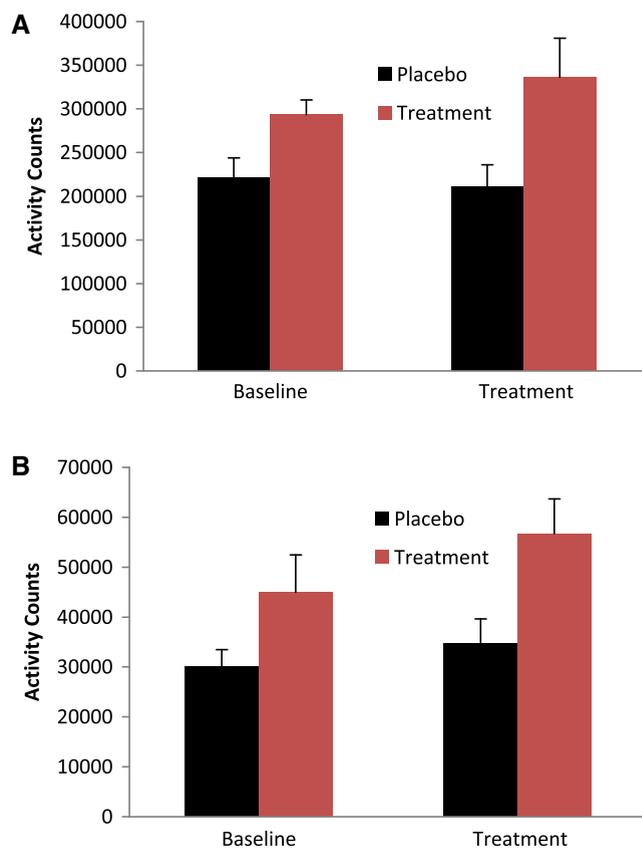
effect on fear of human beings. Although not examined sufficiently in this study, these measures tended to decrease from baseline in placebo-treated fearful dogs, suggestive of a sensitization of fear to human beings that may, at least in part, be reduced by treatment; additional studies with larger group sizes would be required to address this hypothesis. No effects on day or night activity levels or on open-field behavior were seen in the current study, indicating the increase in human interaction was not secondary to motor stimulant effects and also ruling out the possibility of sedative effects. In fact, no side effects of treatment were observed.

Given the limited number of subjects in the current study, the random assignment of subjects to treatment groups may have resulted in nonmatched groups. Although no baseline differences were found between the two groups of fearful animals on measures of human approach and interaction at baseline, there was a suggestion that the placebo group may have been more fearful as evidenced by lower levels of human interaction, but not human approach. Similarly, the groups differed in mean day and night activity levels. Consequently, we are unable to determine whether the positive treatment effects would generalize to more severe cases. Regardless, the absence of baseline differences in time

spent near the human being and the treatment-related increase in this measure supports the overall interpretation of an anxiolytic effect of ANXITANE® tablets.

Conclusion

In the current study, ANXITANE® tablets reduced fear in this subpopulation of laboratory Beagles fearful of unfamiliar human beings. The use of objective behavioral tests afforded us the ability to determine statistically significant treatment effects on fearful dogs regardless of the limited sample size, which was related to the limited availability of dogs fearful of human beings in our colony. Specifically, the ANXITANE® tablets group had significantly higher levels of human approach and interaction than placebo at the treatment time-point. Additionally, no motor stimulant or sedative effects were evident and no other safety concerns were found in the current study. Collectively, the current data indicate that ANXITANE® tablets should benefit dogs that demonstrate fearful behaviors toward unfamiliar human beings, and also support the clinical data for the use of ANXITANE® tablets in treating anxiety-related behaviors.

Acknowledgments

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