Effectiveness of Aqua-acupuncture for Reducing Stress of Canine Patients in Veterinary Clinics

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ABSTRACT

The objective of this study was to evaluate the potential of cyanocobalamin (vitamin B12) aqua-acupuncture (aqua-AP) for mitigating veterinary clinic visit stress in dogs. Twenty dogs with a history of stress behaviors at veterinary clinics were recruited and randomly assigned to either Control Group (sham aqua-AP) or Test Group (aqua-AP at GV-20 and bilateral *An-shen*). Only dogs with an elevated heart rate (HR) or exhibiting a positive stress behavior 10 minutes after arrival were included. The change (baseline to 15 and 30-minutes post-treatment) was compared between the 2 study groups for each stress measurement: HR, salivary cortisol and stress behavior score (average of panting, trembling, vocalizing, pacing, hiding, lip smacking). The study showed behavior score improvement at 15-min and 30-min post-treatment in both groups, however, improvement in the aqua-AP group was double that of the control dogs (61% vs 28% at 15 min, 50% vs 23% at 30 min, respectively). When considering only panting behavior, which dominated the behavioral score, the Test Group improvement when compared to Control Group at 15-min (46% vs 10% decrease, respectively) and 30-min (50% vs 23% decrease, respectively) demonstrated a statistically significant difference between the 2 study groups (p= 0.008 at 15 min and 0.004 at 30 min). In both groups, the study did not find significant changes in either HR or salivary cortisol concentration (SCC), although the HRs were reduced in both groups. This small pilot study suggests vitamin B12 aqua-acupuncture (GV-20, *An-shen*) could be useful for reducing canine visit stress at veterinary clinics.

Key words: cyanocobalamine, aqua-acupuncture, GV-20, *An-shen*, canine stress, behavior score, heart rate, salivary cortisol concentration

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ABBREVIATIONS

Aqua-AP	Aqua-acupuncture
HR	Heart rate in beats per minute
SCC	Salivary cortisol concentration
TCVM	Traditional Chinese veterinary medicine

Visits to veterinary clinics are important to maintain optimal health programs for dogs, however, it is estimated that up to 78.5% of dogs become stressed or fearful in the veterinary clinic.[1] Similar to findings in people known as the "white-coat effect" where 60.3% of patients attending a lipid screening clinic experienced blood pressure increases, dogs have been reported to have significant increases in rectal temperature, pulse rate, panting incidence and systolic arterial blood pressure in the hospital environment compared with home.[2] Stress in the veterinary clinic involving hospitalization may also result in clinical signs or behaviors characterized as frenetic (lip smacking, pacing, panting, spinning, trembling, wet dog shake, whining, yawning), freeze (averting gaze, pinning back ears, whale eye sign) or fractious (growling, lunging, showing teeth, snapping).[3] These physiological and behavioral signs of stress in dogs arriving at a clinic may involve stress from transportation, as well as the clinic environment itself. Changes in exam findings related to stress in dogs may result in misinterpretation of exam findings as well as difficulty performing diagnostics, treatment and hospitalization. This may be followed by even more restraint and further stress for a dog as well as increased bite risk to clinic personnel during handling of the animal.

Traditional methods of reducing stress for dogs entering a veterinary clinic typically involve administration of psychotropic drugs, either prior to admission or injectable sedatives given in the clinic. Typical pharmaceuticals include phenothiazines, benzodiazepines or alpha-adrenergic agonists which may create side effects such as respiratory depression, nausea and prolonged recovery. This can result in the need for additional intensive care, particularly in geriatrics and severely ill patients as well as the need to monitor for adverse reactions and interaction with drugs needed for treatment. All of this can create considerable increased

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cost for the client and mask disease.

Response to sedative drugs can be variable and may be ineffective in reducing stress. Trazodone, a commonly used oral anxiolytic for clinic visits, has been evaluated to determine its effects on behavioral signs of stress in hospitalized dogs. It was concluded that trazodone administration reduced stress related signs and behaviors in hospitalized dogs; however maximum effect was 90 minutes after administration. This suggests the drug would need to be given well in advance of transport to the veterinary clinic.[2] Behavioral changes were tracked in this study with trazodone treatment significantly associated with a reduction in lip licking, panting, and whining at 90 minutes post administration. This suggests reduced cortisol levels (not measured in this study) since increased panting has been associated with the measurement of higher cortisol levels.[4]

Non-pharmacological methods of reducing stress may include petting, aromatherapy, pheromonotherapy and acupuncture.[5-11] These modalities do not have the side effects that drugs have but may vary in their effectiveness or availability. There have been studies assessing some of these methods to reduce stress in dogs in other settings that may suggest beneficial effects in reducing stress in dogs at the veterinary clinic. For example, stressors associated with shelter housing have an effect on canine cortisol levels. A study of cortisol levels measured after repeated petting sessions (petting and massage) of 30 minutes in an animal shelter demonstrated significant decreases in plasma cortisol concentration in shelter dogs.[4] Although it would seem likely that there would be similar findings in a veterinary clinic setting, as far as the author is aware, this has not been investigated. Having a trained staff member performing petting and massage for 30 minutes would not be practical or cost effective for most clinics. An alternate plan would be to have the owner provide the calming petting/massage protocol. Challenges for this would be providing technique training and finding owners that are willing to do this prior to and/or upon arrival at the veterinary clinic. Aromatherapy to reduce stress has been evaluated in a rescue shelter. Lavender and to a lesser degree, chamomile, resulted in behavior suggesting a more relaxed state (dogs spent more time resting and less time moving upon essential oil exposure). Aromatherapy involving lavender was also evaluated for a reduction of travel-induced excitement in dogs. The behaviors of resting, moving, vocalizing and sitting were significantly ($p < 0.001$) affected by olfactory stimulation.[6,7] Both studies measured behavior changes with no physiology endpoints included.

Acupuncture may be used to reduce stress in both humans and animals. There appears, however, to be limited evidence that behavioral and/or physiological changes have been measured in dogs following acupuncture to prove this effect and thus predict its usefulness for stress in the veterinary clinic. Aqua-acupuncture (aqua-AP) has been widely used in traditional Chinese medicine as a method of acupuncture point stimulation and is intended to combine the effect of acupoint stimulation and the effect of the substance injected.[12] It appears to create an effect by local spatial configuration changes within an acupuncture point location, which along with the liquid substrate appears to stimulate neuronal activity.[12] The technique may be performed with a variety of substances which include: normal saline, vitamin B12, the patient's blood, homeopathics, medicinal herbs and various drugs. This modified acupuncture technique emerged in China in the 1950s, is widely used in both humans and animals, and is considered to have a more sustained effect than traditional needle stimulation.[13] Rare adverse events have been reported in people and may be related to contamination during the injection process, therefore, proper aseptic technique is important.[13]

Although aqua-AP with vitamin B12 undiluted or diluted in sterile saline is performed commonly in veterinary practice, there is a lack of studies demonstrating its efficacy. According to Xie et al., aqua-AP is used to lengthen and strengthen acupuncture point stimulation and may be used when the patient is not calm enough for other acupuncture techniques.[14] There are several positive attributes that support the use of aqua-AP with diluted vitamin B12 to relieve stress in dogs visiting the veterinary clinic. It is a simple and inexpensive procedure without likely side-effects, does not require the patient to be restrained once aqua-AP is performed (unlike dry needles) and vitamin B12 is considered a *Qi* tonic which makes it useful for concurrent TCVM Patterns such as Spleen *Qi* Deficiency.[14]

Acupuncture points that are important for sedation in the dog include GV-20, which is on the dorsal midline on a line drawn from the tips of the ears level with the ear canals and *An-shen* (pacify *shen*), bilateral points located at the side of the head, caudal to the base of the ear halfway between *Yi-feng* (TH-17) and *Feng-chi* (GB-20).[14] These acupoints are readily accessible in most patients and are commonly injected with diluted vitamin B12 as a method of reducing canine stress. The objective of this study was to evaluate the potential of cyanocobalamin (vitamin B12) aqua-acupuncture for mitigating acute stress associated with a veterinary clinic visit in dogs as measured by physiologic changes of heart rate (HR) and cortisol levels, along with behavior changes (panting, trembling, vocalizing, pacing, hiding, lip smacking). The study hypothesis was that aqua-acupuncture stimulation of GV-20 and bilateral *An-shen* would reduce acute clinic visitation stress in dogs more effectively than sham vitamin B12 injections.

MATERIALS AND METHODS

Subjects were recruited by the author and her clinic staff from dogs having previously displayed anxiety behaviors (e.g., pant, tremble, vocalize, pace, hide, lip smacking) at various veterinary clinics, including the clinic visited in this study. Criteria for study enrollment included: at least 1 year of age; body weight at least 20 pounds; no known aggression towards owner/agent or veterinary staff; no acupuncture treatment for at least 1 month prior to study start; limited activity and no food for at least 1 hour prior to arrival; no water for at least 10 minutes prior to arrival; no known Cushing's or Addison's Disease; no treatment for anxiety or with exogenous steroids for at least 1 week prior to study start; dogs treated with dexamethasone must be off drug at least 1 month due to high cross reactivity with salivary cortisol. After 10 minutes in the waiting room, dogs that did not exhibit panting, trembling, vocalization, pacing, hiding or lip smacking, and/or elevated HR on admission, were excluded from the study. Owners provided informed consent for their dogs to participate.

Qualified subjects were randomly assigned to either the Control Group or the Test Group, with randomization achieved by a random draw by a third party as each patient arrived for the study. The author, a certified veterinary acupuncturist, provided the real or sham aqua-AP treatment. One study assistant, who was unaware of the subject group assignments, collected all data. The owner/agent was also unaware of which group the dog was in.

Dogs in the Control Group received 2 injections of 1000 mcg undiluted vitamin B12 with a 27g hypodermic needle and syringe[a] into 2 sham acupuncture points on the neck (1 *cun* lateral to GV-14, bilateral), with a total volume of 0.4 mL undiluted vitamin B12 (0.2 mL injected into each sham point). Subjects in the Test Group received aqua-AP with 1000 mcg (undiluted) vitamin B12 diluted according to body weight to obtain an adequate volume to treat the points at GV-20 and bilateral *An-shen*, respectively. The dose of vitamin B12 was thus the same in the Control Group as it was for the Test Group. The acupoint, GV-20, was injected with 0.2 mL diluted vitamin B12 in all size dogs; *An-shen* with 0.5 mL diluted vitamin B12 into each of two points for dogs 20-40 pounds, with 1 mL diluted vitamin B12 into each point if 40-90 pounds and with 1.5 mL diluted vitamin B12 into each point if greater than 90 pounds body weight.

All data collection was done in the afternoon, as recommended by the commercial laboratory performing salivary cortisol testing for individual study dogs. Each of the study subjects was transported by the owner or owner's agent to the clinic from at least 10 minutes away. After arriving, subjects were weighed and then waited (with the owner/agent) in the waiting room for 10 minutes. Individuals being considered for enrollment were confirmed by the assistant that the dog had met all inclusion criteria before being taken to a clinic exam room. In that room, stress behaviors were scored (by the blinded assistant) along with collection of HR and saliva for salivary cortisol within 4 minutes. For each subject, the HR data was determined by auscultation (an average of 2 sets for 15 seconds each) and collection of saliva for salivary cortisol levels was done in 60-90 seconds. Salivary samples were collected as directed by the commercial laboratory[b] running cortisol levels with supplies provided by the company. Each dog's mouth was sampled with a cotton swab[c] (verified by the assistant for adequate wetting by sight or touch) then placed into saliva collection tubes which were immediately frozen. When all samples had been collected, they were shipped as a batch on dry ice to the laboratory[b]. Salivary cortisol concentration (SCC) was measured for each sample in micrograms/dL (in duplicate) and a mean SCC was reported. A dog's stress behavior score which included 6 assessments (panting, trembling, vocalizing, pacing, hiding, lip smacking) were assessed by the same blinded assistant who observed/graded behaviors through a window while standing outside the room. The stress behaviors were graded on a sliding scale from 0 (behavior not exhibited) to 3 (behavior continuously exhibited): 0 = no stress, 1 = mild stress, 2 = moderate stress and 3 = severe stress.

After baseline data collection, the principle investigator (HM) immediately entered the exam room to perform the aqua-AP treatment with the owner's assistance. The treatment took less than 1 minute and the investigator then left the room. The dog stayed with the owner in the exam room and the owner was instructed to sit quietly (reading or other quiet activity) and not interact with the dog. All subsequent post-treatment data collections which occurred 15 and 30 minutes after the treatment remained in that same room. Each was performed within 4 minutes, using the same method as that used to take the baseline data.

Statistical analysis consisted of a treatment effect comparison between the control and test groups. It was performed with respect to the change (post-treatment as compared to baseline) in each of the stress outcome measurements: HR, SCC, overall behavior score (sum of all 6 stress behaviors). Without assuming normality of the data, nonparametric Wilcoxon Rank Sum test was employed to test the null hypothesis that the improvement of a stress measurement in the Test Group population is the same as that in the Control Group population against the alternative that the improvement of a stress measurement in the Test Group population is greater than in the Control Group population.

If the study was able to recruit and enroll a total of 20 dogs with 10 dogs in each study group after the randomization procedure, this sample size (assuming probability of a test subject having greater improvement than a control subject was 80%) would have approximately 80% power to reject the null hypothesis with a significance level of 0.05 (Wilcoxon Rank Sum test). All statistical analyses were done using commercial statistical software[d].

RESULTS

There were 20 dogs meeting inclusion criteria that were enrolled in the study with dogs assigned by randomization to each study group. All enrolled dogs completed the trial and all data were included in the statistical analyses. The study subjects included a variety of breeds. There were a total of 9 breeds among the 10 dogs in the Test Group: Australian Shepherd Mix, Pit Bull (2), Vizsla, German Shepherd Mix, Finnish Lapphund, German Shepherd, Airedale, Golden Retriever, and

Greater Swiss Mountain Dog. Among the 10 dogs in the Control Group, there were also 9 breeds: Labrador Retriever (2), German Shepherd, Pit Bull, Wirehaired Pointer Mix, Beagle, Bull Mastiff, Border Collie, Australian Shepherd, and Airedale. Neither study group was dominated by a particular breed. Analyses on a subject's signalment data (sex, age, body weight) did not find statistically significant group differences (p = 1.00, 0.20, and 0.97, respectively). Additional analyses also found no evidence of significant group differences with respect to transportation time (p = 0.97) or previous experience at the clinic (p = 1.00), which could have affected the subject's stress level.

Table 1: Summary statistics of heart rate data. Both groups had statistically significant decreases when compared to their baseline data, but the Control Group and Test Group were not significantly different from each other.

Heart Rate	Control		Test		*p*-value (Test vs. Control)
	Mean±SD	Median (range)	Mean±SD	Median (range)	
Baseline	101.8±14.5	107 (78, 122)	105.5±18.7	111 (76, 128)	0.591
Change 15-mins Post-treatment	-8.8±10.7 (↓ 8.6%)	-13 (-22, 10) (↓ 12.1%)	-9.8±12.3 (↓ 9.3%)	-8 (-34, 8) (↓ 7.2%)	0.610
	vs. baseline: $p = 0.037$*		vs. baseline: $p = 0.027$*		
Change 30-mins Post-treatment	-16.8±15.3 (↓ 16.5%)	-18 (-46, 8) (↓ 16.8%)	-14.6±13.0 (↓ 13.8%)	-12 (-34, 10) (↓ 10.8%)	0.651
	vs. baseline: $p = 0.010$*		vs. baseline: $p = 0.010$*		

*Statistically significant $p < 0.05$

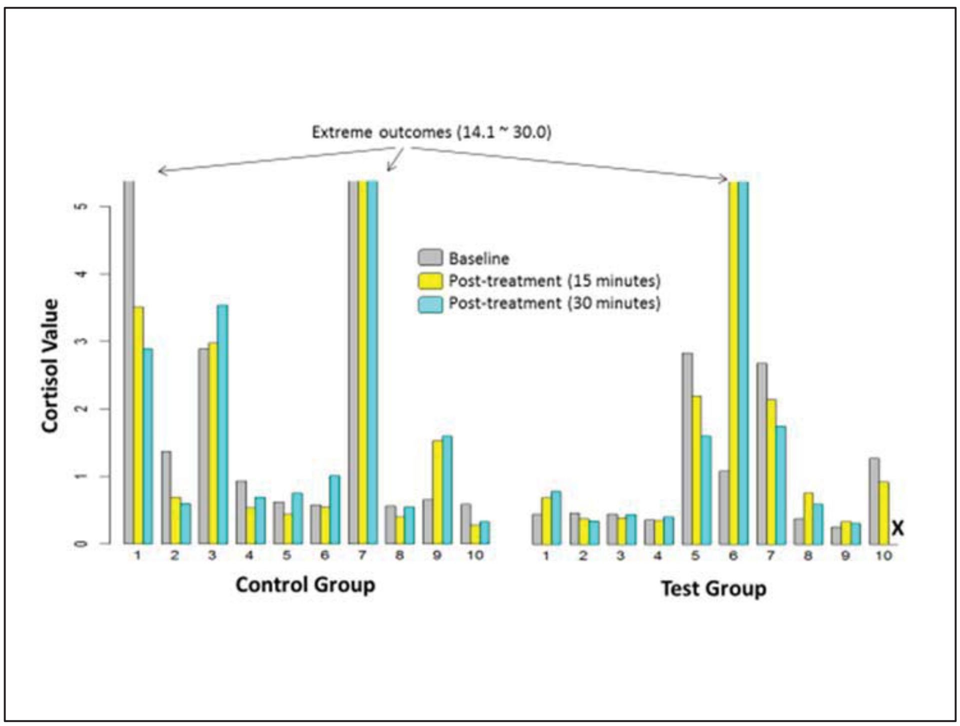

Figure 1: Salivary cortisol concentration (SCC) data for each individual subject from samples collected at baseline, 15-minutes and 30-minutes post-treatment. Individual SCC values clearly exhibit significant data variation among subjects, even within the same treatment group.

For each subject, HR data was measured during the baseline period and at 15- and 30-minutes (min) post-treatment, respectively. During the baseline period, the difference in median HR between the two groups was not statistically significant (p = 0.591). For both groups (8/10 dogs), the median HR decreased at 15-min post-treatment time and then again reduced after another 15 min (i.e., 30-min post-treatment). Based on the Wilcoxon Sign Rank test, for both groups, the median HR reduction at 15-min post-treatment was significant (p = 0.037 and 0.027 in control and test groups, respectively), and was also significant at 30-min post-treatment (p = 0.010 for both groups). Comparison of the post-treatment HR reduction in test and control groups at 15 minutes (p = 0.610) and 30 minutes (p = 0.651) demonstrated the Test Group was not significantly different from the Control Group (Table 1).

The individual salivary cortisol concentration (SCC) values clearly exhibited significant data variation among subjects, even within the same treatment group (Figure 1). Seven out of 10 subjects in the Control Group had reduced SCC at the 15-min post-treatment time compared to the baseline value, but after 30 min, only 5 remained smaller than the baseline values. In the Test Group, 6 out of the 10 dogs showed reduced SCC at the 15-min post-treatment time, and at 30-min 4 out of 9 (missing data in 1 Test subject due to insufficient saliva at the 30 min collection) remained smaller than the baseline values. The reduction was not significant for the Control Group (p = 0.43 and 0.84) or Test Group (p = 0.85 and 0.82) at 15-min and 30-min sampling times, respectively (Table 2).

Table 2: Summary statistics of salivary cortisol concentration data. Unexpectedly, the Test Group median SCC values at both 15 and 30 minutes after treatment were larger than baseline values and the Control Group median SCC values dropped at 15 minutes but then increased to above baseline values at 30 minutes. Differences in median SCC changes between the two treatment groups were not statistically significant.

Salivary Cortisol Concentration	**Control**		**Test**		***p*-value (Test vs. Control)**
	Mean±SD	**Median (range)**	**Mean±SD**	**Median (range)**	
Baseline	3.89±6.08	0.79 (0.57, 15.59)	1.03±0.97	0.46 (0.26, 2.83)	0.075
Change 15-mins Post-treatment	-1.08±3.76 (↓ 27.8%)	-0.18 (-11.6, 1.60) (↓ 22.8%)	2.79±9.18 (↑ 271%)	-0.03 (-0.63, 28.9) (↓ 6.5%)	0.759
	vs. baseline: p = 0.43		vs. baseline: p = 0.85		
Change 30-mins Post-treatment	-0.33±4.90 (↓ 8.5%)	+0.06 (-12.2, 8.06) (↑ 7.6%)	1.12±4.51 (↑ 109%)	-0.002 (-1.28, 13.0) (↓ 0.43%)	0.330
	vs. baseline: p = 0.84		vs. baseline: p = 0.82		

Table 3: Summary of behavioral assessments recorded on a sliding scale from 0 (behavior not exhibited) to 3 (behavior exhibited continuously) for Control Group at baseline; then at 15 and 30 minutes after treatment.

Control Dog No.	**Panting Score**			**Trembling Score**			**Vocalization Score**			**Pacing Score**			**Hiding Score**			**Lip Smacking**		
	0	**15'**	**30'**	**0**	**15'**	**30'**	**0**	**15'**	**30'**	**0**	**15'**	**30'**	**0**	**15'**	**30'**	**0**	**15'**	**30'**
1	2	2	2	0	0	0	1.5	1	1.5	0.5	0	0	0	0	0	0	0	0
2	2	1.5	1.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	2.5	2	1	1.3	1	0.5	0	0	0	0	0	0	0.8	1	0.8	0.6	0.3	0.3
4	0.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0.8	0.8	0	0
5	0.6	0.8	0.5	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
6	1.9	1.5	1.0	0.7	0	0	0.6	1	1	0.5	0.4	0.4	0	0	0	0.6	0	0
7	1.9	0.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0.6	0	0
8	2.5	2.6	2.6	0	0	0	0	0	0	2.4	1.7	1.8	0	0	0	1	0.8	0.9
9	2.3	2.8	2.5	0.6	0.4	0	0	0	0	1.1	2	1.7	0	0	0	1.2	0.9	1.4
10	1	1	0.5	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0

Yellow = no change; Pink = improved; Cyan = worsened

Table 4: Summary of behavioral assessments recorded on a sliding scale from 0 (behavior not exhibited) to 3 (behavior exhibited continuously) for Test Group dogs for baseline; then at 15 and 30 minutes after treatment.

Test Dog No.	Panting Score			Trembling Score			Vocalization Score			Pacing Score			Hiding Score			Lip Smacking		
	0	15’	30’	0	15’	30’	0	15’	30’	0	15’	30’	0	15’	30’	0	15’	30’
1	2	2	1	1	0	0	2	0	0	1	0	0	0	0	0	1	1	0
2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
3	2.6	0.8	0.3	0	0	0	0	0	0	2	0	0	0	0	0	2	0	0
4	2.2	0.8	1.3	0	0	0	0	0	0	0	0	0	0.8	0	1.5	0.3	0	0
5	1.7	0.8	0.8	0	0	0	1.5	0	0	0	0	0	0	0	0	0	0	0
6	3	2.8	2.3	0	0	0	0	0	0	0	1.4	1.3	2	0	1.2	0.6	1	0.3
7	0.6	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0.9	0.6	0	0	0	0	0	0
9	2.1	1.6	1.7	0	0	0	0	0	0	0	0	0	1	0.7	0.8	1	0.6	0
10	2.2	0.9	0	0	0	0	0	0	0	0.8	0	0	0.8	0.9	0.9	0.8	0	0

Yellow = no change; Pink = improved; Cyan = worsened

Due to the SCC value outliers in each of the groups and at each sampling time, statistical analyses were focused on median values (Table 2). During the baseline period, the median SCC values were 0.79 (range: 0.57~15.6) and 0.46 (range: 0.26~2.83) for the control and test groups, respectively. This difference was not statistically significant ($p = 0.075$). Unexpectedly, the Test Group median SCC values at both 15 and 30 min after the treatment were larger than that during the baseline. The Control Group median SCC values dropped at 15 min but then increased to above baseline values at 30 min. Differences in median SCC changes between the two treatment groups were not statistically significant ($p = 0.759$ and 0.330 for 15-min and 30-min sampling times, respectively).

There were 6 stress behaviors (panting, trembling, vocalizing, pacing, hiding, lip smacking) that were evaluated in study dogs at baseline and at 15 and 30 min after treatment. All behaviors were graded by an assistant blinded to the study group on a sliding scale of 0 (behavior not exhibited) to 3 (behavior continuously exhibited). There was variability in frequency of behaviors with some exhibited only rarely in Test Group dogs such as trembling (1 dog at baseline) and vocalization (2 dogs only at baseline) and other behaviors such as panting present at multiple timepoints in all study dogs but one (Tables 3 and 4). The panting score with its observed consistency dominated the other behaviors when calculating the overall treatment effects. In the Test Group, improvements in panting at both 15-min and 30-min were significant ($p = 0.008$ and 0.004, respectively) whereas the Control Group improvement was not significant at 15 min ($p = 0.203$), but did improve to significance after the dogs had been sitting quietly at the 30-min assessment ($p = 0.023$). Comparison of improvement between the 2 groups demonstrated a significantly greater effect for the Test Group at both 15 and 30 min ($p = 0.021$ and $p = 0.031$, respectively) for diminished panting behavior (Table 5, Figure 2).

The overall behavior score was calculated as the sum of all 6 stress behaviors scores. Based on the Wilcoxon Rank Sum test, the groups were comparable with no statistically significant difference in baseline median score ($p = 0.739$) (Table 6). In both groups, the median behavior score decreased and improvements were greater at 30 min than at 15 min post-treatment with improvement in the Test Group double that of the control dogs (61% vs 28% at 15 min, 50% vs 23% at 30 min, respectively) (Table 6, Figure 3). The improvements in both groups at 15 mins ($p = 0.020$ both groups) and 30 mins ($p = 0.004$ and 0.008 for the control and the test groups, respectively) was statistically significant but the 2x greater improvement in the Test Group was not large enough in this small study to achieve statistical significance when compared to the Control Group ($p = 0.083$ for 15 min, $p = 0.127$ for 30 mins) (Table 6).

DISCUSSION

The importance of low stress handling of dogs when visiting veterinary clinics is being increasingly recognized by veterinarians as an important consideration in the daily conduct of medical treatments. In this randomized controlled blinded clinical study, 20 dogs demonstrating acute clinic visitation stress were divided into 2 groups (sham vs aqua-acupuncture) to evaluate effectiveness of the test procedure. The study compared change from baseline for behavioral characteristics (panting, trembling, vocalizing, pacing, hiding, lip smacking) and physiologic parameters (heart rate, cortisol level).The behavior score consisting of the 6 stress behaviors improved at 15-min and 30-min post-treatment in both groups, however, improvement in the Test Group was double that of the control dogs (61% vs 28% at 15 min, 50% vs 23% at 30 min, respectively), although not reaching statistical significance in this small study. When considering only panting behavior, which dominated the behavioral score, the Test Group improvement when compared to Control Group at 15-min (46% vs 10% decrease, respectively) and

30-min (50% vs 23% decrease, respectively) demonstrated a statistically significant difference between the 2 study groups (p = 0.008 at 15 min and 0.004 at 30 min).There was marked individual variability of physiologic parameters in both study groups with no significant treatment-associated changes found for HR or salivary cortisol concentration (SCC). Study findings partially supported the hypothesis with statistically significant reduction of stress behaviors but not for physiologic changes of heart rate and cortisol changes.

Animals respond to stress by activating a wide array of behavioral and physiologic responses with stress defined as any stimulus that disrupts the body's internal balance. Traditional Chinese medicine emphasizes balance, particularly the balance between "*Yin* and *Yang*".[11] Translated to conventional western medicine, an important effect of acupuncture is to modulate the imbalance between parasympathetic and sympathetic activity (autonomic nervous system) and/or neuroendocrine changes (hypothalamic-pituitary-adrenal

Table 5: Summary statistics for panting data. The Test Group had a statistically significant greater improvement at both the 15- and 30-minute assessments when compared to the Control Group.

Overall behavior score	Control		Test		p-value (Test vs. Control)
	Mean±SD	Median (range)	Mean±SD	Median (range)	
Baseline	1.76±0.67	1.95 (0.63, 2.50)	1.94±0.76	2.13 (0.56, 3.00)	0.562
Change 15-mins Post-treatment	-0.30±0.58 (↓ 17.0%)	-0.19 (-1.50, 0.50) (↓ 9.7%)	-0.86±0.60 (↓ 44.3%)	-0.97 (-1.83, 0.00) (↓ 45.5%)	0.021*
	vs. baseline: p = 0.203		vs. baseline: p = 0.008**		
Change 30-mins Post-treatment	-0.60±0.71 (↓ 34.1%)	-0.50 (-1.90, 0.25) (↓ 25.6%)	-1.12±0.67 (↓ 57.7%)	-0.97 (-2.31, -0.41) (↓ 45.5%)	0.031*
	vs. baseline: p = 0.023*		vs. baseline: p = 0.004**		

Statistically significant *p < 0.05; **p < 0.01

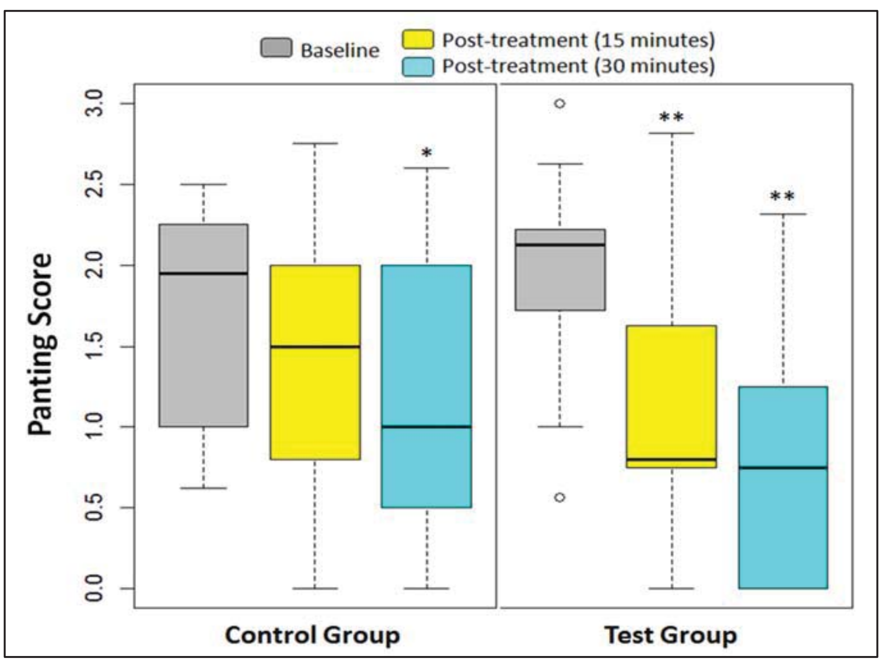

Figure 2: Panting behavior score from each subject group assessed during baseline, 15-minute, and 30-minute post-treatment times. Circles above and below the whiskers are outliers. Values indicated at the top of the box by *p<0.05 or **p<0.01 differ significantly when compared to the baseline median (horizontal line inside each box).

Table 6: Summary statistics of overall behavior score data. The median behavior score significantly decreased at 15 and 30 minutes for both groups, however, improvement in the Test Group was double that of the controls (61% vs 28% at 15 minute, 50% vs 23% at 30 minute, respectively); decreased behavior score = improved stress level.

Overall Behavior Score	Control		Test		*p*-value (Test vs. Control)
	Mean±SD	Median (range)	Mean±SD	Median (range)	
Baseline	3.44±1.65	3.26 (1.63, 5.88)	3.70±2.38	3.70 (0.00, 7.00)	0.739
Change 15-mins Post-treatment	-0.85±0.84 (↓ 24.7%)	-0.91 (-2.13, 0.84) (↓ 27.9%)	-1.99±20.3 (↓ 53.8%)	-2.24 (-5.83, 0.88) (↓ 60.5%)	0.083
	vs. baseline: $p = 0.02$*		vs. baseline: $p = 0.02$*		
Change 30-mins Post-treatment	-1.09±1.00 (↓ 31.7%)	-0.75 (-2.70, 0.41) (↓ 23.0%)	-2.33±0.49 (↓ 63.0%)	-1.83 (-6.31, 0.56) (↓ 49.5%)	0.127
	vs. baseline: $p = 0.004$**		vs. baseline: $p = 0.008$**		

Statistically significant *p <0.05 or **p <0.01

axis, HPA) of the nervous system.[11] Its sensory stimulation affects several classes of afferent nerve fibers and once directed to the spinal cord and then higher centers in the central nervous system (CNS), it triggers neurophysiological events that generate therapeutic outcomes.[11] The stimulation of acupoints, therefore, can prevent stress-induced changes in the autonomic nervous system and the HPA axis.

Physiologic measurements of stress in the current study investigated both the autonomic nervous system response (heart rate) and neuroendocrine changes of HPA axis (salivary cortisol). Activation of the autonomic nervous system during a stress event quickly promotes physiological changes through synaptic transmission by its 2 branches: parasympathetic and sympathetic nervous system.[15] The release of norepinephrine and stimulation of sympathetic preganglionic neurons creates an increased heart rate (i.e. sympathetic stimulation, "fight or flight") or conversely acetylcholine from parasympathetic postganglionic receptors decreases heart rate (i.e. calm restorative state). The balance between the two is sympathovagal modulation. It was hypothesized that study animals exposed to clinic visitation stress would first undergo sympathetic stimulation with increased heart rates. After removal of the stress, decreased heart rates would occur with a greater decrease in the aqua-AP treated dogs. An unexpected study finding was that although both groups had statistically significant heart rate decreases at both the 15 and 30 minute measurements, there was a mild trend to a greater effect in the controls when compared to test dogs at 15 minutes (12.1% ↓ vs 7.2% ↓, respectively) and 30 minutes (16.8% ↓ versus 10.8% ↓). This non-finding is similar to inconsistent results in a number of human studies examining the association between perceived stress and cardio-autonomic changes. Those studies varied between positive association, negative association or lack of association. Possible explanations included different effects depending on how the stress was perceived and measurement limitations (how measured, frequency, timing of measurement).[15]

HPA axis activity can be assessed using salivary cortisol sampling. Cortisol plays a role in augmenting the activity of the autonomic nervous system such as enhancing the sympathetically mediated cardiovascular response to stress. It is released in a circadian pattern with the greatest levels just after awakening and gradual decline throughout the day. Similar to the present study, although measured frequently in humans to evaluate stress, a lack of uniform findings in relation to cortisol levels is often the case.[15,16] Similarly, although salivary cortisol concentration (SCC) has been used in behavioral studies for dogs, its normal canine range for stress levels has not been established. Hekman et al. noted SCC should not be considered alone as a predictive measurement of stress, despite it being used commonly.[16] The study pointed out there is marked individual variation, similar to the present study and it is unclear as to how long salivary cortisol lasts after an event or if it continues to be produced for a period of time. It is unknown if an intervention such as aqua-acupuncture might affect SCC for a short period of time. It is thought that salivary cortisol starts to rise 5 minutes after a stressor and may take up to 20 minutes to reflect that stress. There is also debate as to timing of its return to baseline which has been projected at anywhere from 40 to 60 minutes.[17] In retrospect, continued SCC sampling every 15 minutes for at least an hour post-treatment may have provided more consistent data. Other factors to consider in a larger study to produce more optimal data might include: bring dogs into the clinic at a busier time of day to increase stress exposure, bring all dogs in with their owner (versus agent) to reduce variation among subjects and have all dogs naive to the clinic, researcher, and assistant.

The behavioral outcome data supported stress behavior modification in aqua-AP treated study dogs. Findings demonstrated behavior score improvement at 15-min and 30-min post-treatment in both groups, however, improvement in the aqua-AP group was double that of the control dogs (61% vs 28% at 15 min, 50% vs 23% at 30 min, respectively). In addition, when considering the panting behavior, which dominated the behavioral score, the Test Group improvement when compared to Control Group at 15-min (46% vs 10% decrease, respectively) and 30-min (50% vs 23% decrease) demonstrated a statistically significant difference between the 2 study groups. This would suggest reduced cortisol levels subsequent to aqua-AP treatment since increased panting has been associated with the measurement of higher cortisol levels; even though under the methods of this study it was unable to be demonstrated.[4] Fong et al. demonstrated similar positive behavior modification findings when comparing dry needle acupuncture stimulation of *An-shen* to butorphanol for the calming of dogs undergoing a short veterinary procedure.[18]

Anatomic inspection of the 2 acupoints used in this study strongly suggest why the behavioral data supported aqua-AP associated changes in the test dogs when compared to controls. The acupoint, GV-20, is surrounded by sensory fibers provided by the greater occipital nerve (cervical nerve 2, dorsal branch). Information from stimulation of this area is carried along the spinocervicothalamic pathway to contralateral thalamic neurons and on to the cerebral cortex.[19] The intersection with the thalamus is important as the neurons that reside there serve busy unique roles, ranging from relaying sensory and motor signals to regulation of consciousness and alertness which underscores the benefit of GV-20 for behavior modulation and stress relief.

The second acupoint used, *An-shen*, has even greater anatomical complexity. This anatomic site contains 2 cervical nerves and 4 cranial nerves (VII-facial, X-vagus, IX-glossopharyngeal, XI-accessory) coursing through the area.[19] Projections from the cranial cervical ganglion are numerous and many of the axons leaving the ganglion create plexi associated with a number of arteries including the external and internal carotids which carry sympathetic information into the head. Of interest, the cranial nerves VII, IX, X travel to the solitary tract nucleus from their sensory ganglia. Excitement of nerves in this area stimulate input into the brain and, in humans, it has been documented this area receives inputs that mediate the carotid sinus reflex, aortic chemoreceptor reflexes, several respiratory reflexes and reflexes within the gastrointestinal system (motility, secretion). In addition, it is important to consider the presence of the vagus nerve in this area as vagus nerve axons from the nucleus ambiguous provide parasympathetic modulation to the heart which assists balancing increased sympathetic stimulation in dogs undergoing “fight or flight” during clinic stress.[19]

In summary, based on the findings of this randomized controlled blinded study, aqua-acupuncture with vitamin B12 at acupoints GV-20 and bilateral *An-shen* may be useful in a veterinary clinic setting to

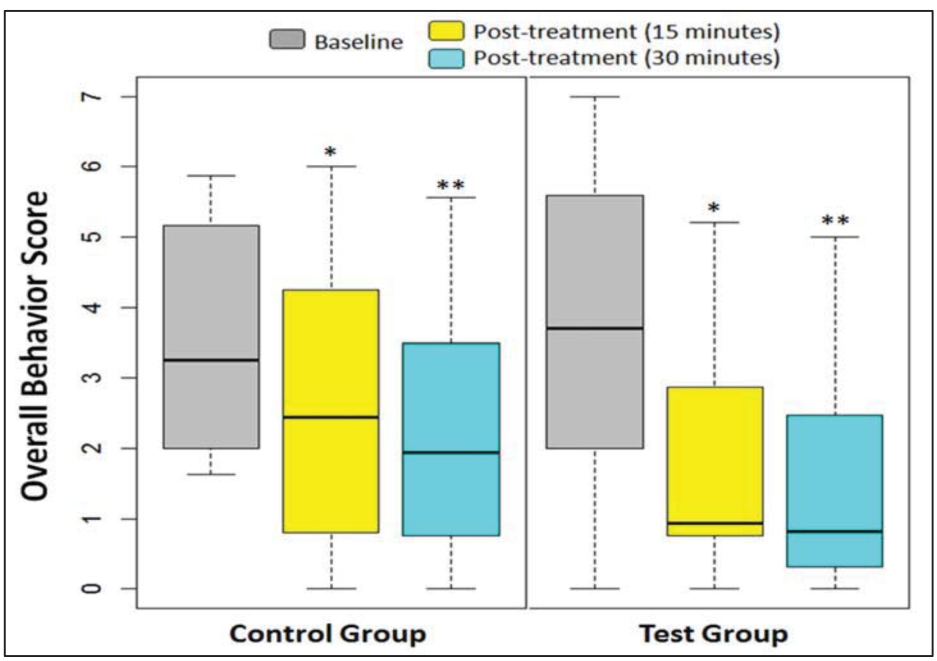

Figure 3: Overall behavior score data from each subject group assessed during baseline, 15-minute, and 30-minute post-treatment times. Values indicated at the top of the box by *p<0.05 or **p<0.01 differ significantly when compared to the baseline median (horizontal line inside each box).

reduce stress in dogs before further diagnostics, treatment, or hospitalization occurs. This technique is simple, inexpensive, and with minimal safety concerns in a clinical setting. In the future, larger, blinded prospective studies with a more active environment presenting additional stressors is needed to support the findings of this small study.

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Declaration of Interest

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FOOTNOTES

[a] Monoject standard needles and syringes; Kendall (Covidien) products, Mansfield, MA, USA

[b] SalivaLab, Salimetrics Biotechnology Co, Carlsbad, CA, USA

[c] SalivaBio Children's Swab, Saliva collection device, Salimetrics Biotechnology, Carlsbad, CA, USA

[d] R version 3.5.2. The R Foundation for Statistical Computing, Vienna Austria; http://www.R-project.org

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