ANIMAL COMPONENT OF RESEARCH PROTOCOL (ACORP)
Main Body
VERSION 4

See Instructions for Completion of the Animal Component of Research Protocol (ACORP Instructions), for help in completing specific items.

A. **ACORP Status.**

1. Full Name of Principal Investigator(s)► [redacted]
   Co-PIs: [redacted]

2. VA Station Name (City) and 3-Digit Station Number► **Richmond 652**

3. Protocol Title► **Nanoparticle Injection into Ganglionated Neural Plexi to Prevent Atrial Fibrillation**

4. Animal Species covered by this ACORP► **Canines**

5. Funding Source(s). Check each source that applies:

   ►( ) Department of Veterans Affairs.
   ►( ) US Public Health Service (e.g. NIH).
   ►( ) Private or Charitable Foundation -- Identify the Foundation:
   ►( ) University Intramural Funds – Identify the University and Funding Component:
   ►( ) Private Company – Identify the Company:
   ►(X) Other – Identify Other Source(s): **Commonwealth of Virginia**

6. Related Documentation for IACUC reference.

   a. If this protocol applies to a project that has already been submitted to the R&D Committee for review, identify the project:

      (1) Title of project► Nanoparticle Injection into Ganglionated Neural Plexi to Prevent Atrial Fibrillation

      (2) If approved by the R&D Committee, give the date of approval► **TBD**

   b. Triennial review. If this protocol is being submitted for triennial *de novo* review, complete the following:

      (1) Identify the studies described in the previously approved ACORP that have already been completed
      ►

      (2) Indicate the numbers of animals of each breed/strain/genotype that have already been used, and adjust the numbers shown in Item I accordingly
      ►

      (3) Describe any study results that have prompted changes to the protocol, and briefly summarize those changes, to guide the reviewers to the details documented in other Items below.
      ►

c. List any other relevant previously approved animal use protocols (copy the lines below as needed for each protocol listed).

(1) Title of other protocol ►
(2) IACUC approval number of other protocol ►
Give the name of the VA station or other institution that approved it, if it was not approved by the IACUC that will review this ACORP ►

7. Indicate the type(s) of animal use covered by this protocol (check all that apply):

►(X) Research
►( ) Teaching or Training
►( ) Testing
►( ) Breeding and colony management only; not for any specific research project
►( ) Holding protocol (as specified by local requirements; not required by VA, PHS, or USDA)
►( ) Other. Please specify►

Proposal Overview

B. **Description of Relevance and Harm/Benefit Analysis.** Using non-technical (lay) language that a senior high school student would understand, briefly describe how this research project is intended to improve the health of people and/or other animals, or otherwise to serve the good of society, and explain how these benefits outweigh the pain or distress that may be caused in the animals that are to be used for this protocol.
►

Atrial Fibrillation (AF) is an abnormal rhythm in the heart where the top chambers of the heart, the atria, beat extremely fast, also known as fibrillation. It is the most common cardiac arrhythmia worldwide in humans. It is a major epidemic responsible for increased risk of stroke, heart failure, hospitalizations and death. AF occurs in up to 20-30% of patients that have undergone open-heart surgery which is significantly higher than the normal population. These patients are then subjected to prolonged hospital stays, with an additional estimated in-hospital expenditure of $14,000 per patient, $57 million per annum in Virginia alone, and over a billion dollars worldwide.

Current therapies for postoperative AF consist of pre-operative use of medicines known as beta-blockers (a class of drugs that are particularly used to manage cardiac arrhythmias, and to protect the heart from a second heart attack after a first heart attack). However, this therapy has variable success rates. With the increasing epidemic of AF there is a growing need for novel therapies that are more effective at preventing or treating it in postoperative and other patients. One line of potential therapies seeks to disrupt cardiac nerves that are known to cause AF. These nerves are organized along the outside surface of the atria in clusters known as ganglionic plexi (GP). Current efforts to block the signals from these nerves utilize radio frequency ablation to destroy the GP. However, the usefulness of this technique is limited by the simultaneous damage to the adjacent cardiac muscle of the atria. Recently, our group, in collaboration with the other investigators on this grant, has demonstrated that injection of botulinum toxin (botox) into GPs can decrease the incidence of postoperative AF.

In this project we aim to determine if continuous release of either botox or calcium chloride (CaCl2) from nanoparticles (microscopic inorganic particle that can be used for transport) injected into selected GP can prevent AF in a postoperative AF canine model. This therapeutic strategy is particularly promising because it will spare the adjacent structures preventing atrial scar tissue which is known to be linked to arrhythmias as well. The dogs used in the project are chosen because of their proven similarities to human cardiac conduction. These experiments would be impossible in smaller animals because of their faster heart rates and inherent resistance to AF. The animals involved in this study will be anesthetized for surgery, and further data

collection should not cause any perceivable pain. All animals will be humanely euthanized at the end of the study.

C. **Experimental Design.**

1. **Lay Summary**. Using non-technical (lay) language that a senior high school student would understand, summarize the conceptual design of the experiment in no more than one or two paragraphs.
►

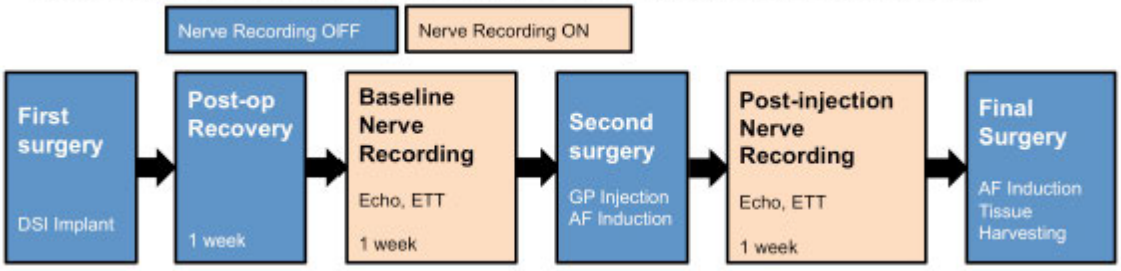
In this project, we aim to determine if we can inhibit the incidence and inducibility of atrial fibrillation in a novel post-operative model of AF. We are using a chronic survival AF model because of the relative increased incidence of AF in this setting. We will perform an initial thoracotomy (surgery involving an incision into the chest wall) surgery on the animals to implant our autonomic nerve activity recording device (Data Science International) into the left sided GPs and the left stellate ganglion (LSG) a large center of nerve activity in the left chest that controls most of the autonomic nerve activity going to and from the heart. During this procedure we will expose the outside (epicardial) surface of the left atrium. We will perform an electrophysiology (EP) study to induce AF. The AF inducibility results will therefore act as our baseline against which to compare results from repeated EP studies later. Once the animals have recovered from the surgery (approximately 7-10 days), we will begin recording autonomic activity, along with electrocardiograms (process of recording the electrical activity of the heart over a period of time using electrodes placed on the skin) and echocardiograms (a type of ultrasound test that uses high - pitched sound waves that are sent through a device called a transducer) to establish the autonomic nerve activity, cardiac electrical activity and cardiac function in the postoperative heart. After at least 21 days of recovery from first surgery, we will perform a second survival thoracotomy surgery. During this surgery we will perform an electrophysiology (EP) study where we attempt to induce atrial fibrillation in the animals to establish a baseline for AF inducibility during postoperative state in all groups..
This will be compared with the baseline state during first surgery. This will be followed immediately by microinjection of nanoparticles releasing neurosuppressants consisting of either botox or CaCl into either 2 (left sided) or 4 (bilateral) GPs. After administration of therapy, we will perform a repeat electrophysiology study to induce AF. AF inducibility will be compared between baseline first surgery vs second surgery prior to and after therapy. The animals will be recovered from surgery. We will continue to monitor these animals by recording autonomic nerve activity, cardiac electrical activity, and cardiac function as before for one week. The animals will undergo final surgery 7 days after second surgery. During this surgery, we will then attempt to induce AF with an EP study, in all groups to determine what effect the various treatments have on prevention of postoperative AF. After this attempt, all animals will be euthanized so that we can harvest the heart for histology to look at the location of injected nanoparticles in the respective GPs, and determine if there is associated neuronal cell death.
This model represents the first survival model of postoperative AF with two survival thoracotomies. It also represents the first ever attempt at autonomic modulation by injection of nanoformulated neurosuppressants into cardiac ganglionated plexi. Thus, the experimental protocol is a pilot protocol with the potential for unexpected events.

The rationale for using a survival thoracotomy approach is so that the effect of therapy on suppressing AF and autonomic nerve activity can be compared before and at various stages after therapy. Each subject acts as its own internal control. This model also represents a true depiction of the cardiac postoperative state for human cardiac surgery, and is therefore a novel and robust model for the study of postoperative AF.

2. **Complete description of the proposed use of animals.** Use the following outline to detail the proposed use of animals.

a. **Summarize** the design of the experiment in terms of the specific groups of animals to be studied. ►

Figure 1. Experimental Protocol. Events occur in sequence from left to right.



1. We will use 20 Mongrel canines (25-30kg each). There will be 3 groups. Group 1 (N=5) will be calcium chloride only control (injection of calcium chloride alone without nano-formulation), Group 2a (N=5) will have injection of nano-formulated calcium chloride into all 4 GPs, Group 2b will have injection of nano-formulated calcium chloride into 2 GPs including sentinel GP (N=5), and Group 3 will have injection of nano-formulated botulinum toxin (botox) into all 4 GPs. Each dog will act as its own negative control (prior to neurotoxin injection) by virtue of inducing AF prior to and after (1 hour and 1 week) after neurotoxin injection.

2. There will be three sequential surgeries per animal. First and second surgeries are survival surgeries, followed by third (final) surgery.

   -First surgery will involve implantation of DSI recording electrodes.

   -Second surgery (at least 21 days later) will involve attempts to induce AF, followed by injection of neurotoxins into GPs, followed by another repeat attempt at AF induction.

   -Third and final (euthanasia) surgery (at least 7 days after second surgery) will involve induction of AF, euthanasia and tissue harvesting.

3. The rationale for this design is as such:

   a. Survival surgeries will allow each animal to be its own internal control, so we can compare GP nerve activity (NA), AF inducibility before, immediately after, and 1 week after GP injection.

   b. The duration of the chronic study was chosen as it is the optimal window for occurrence of postoperative AF (around hours to the first week), and also for peak effect of botox, which is around 3 days, and the peak effect of calcium chloride, which is approximately 1 week.

   c. AF induction 1 hour (during second surgery) and 1 week (during final surgery) after GP injection will allow us to determine whether the effects of nano-formulated neurosuppressant are sustained compared to neurosuppressant alone, and compared with no neurosuppressant at all (internal negative control).

   d. Our chronic model will also allow us to evaluate safety of GP injection by performing studies at baseline and after GP injection. These studies include: (a) echocardiography to evaluate

ejection fraction (EF) (heart function),and rule out potential complications such as pericardial effusion; (b) 9-minute exercise tolerance test (ETT) to evaluate adequate peak heart rate, heart rate recovery functional capacity after GP injection. (c) ECG to assess the safety of potential complications including PR prolongation, QT prolongation and significant bradyarrhythmias. Autonomic nerves are important in regulating cardiac response to stress by increasing heart rate in response to exercise. Suppressing autonomic nerve function can adversely affect exercise capacity by reducing peak heart rate response and prolonging heart rate recovery after exercise. Previous studies have demonstrated significant cardiac autonomic nerve suppression caused by GP ablation or GP botox injection, without adversely affecting exercise capacity. This is most likely because of direct stimulation of catecholamine receptors in the heart by circulating catecholamines. These safety studies are necessary to demonstrate clinical feasibility. They are further detailed in methods. Please note that the ETT studies will only be performed after the dog has completely recovered from prior surgery and is fully ambulant. Hence we typically perform the ETT at least 6 days after surgery.

b. **Justify the group sizes and the total numbers of animals requested.** A power analysis is strongly encouraged; see ACORP instructions.
►
Because of internal comparisons where each animal is its own control, we will have 4 groups. Each group will have 5 animals.

Group 1: injection of calcium chloride without nanoformulation into 2 left sided GP
Group 2: injection of nanoformulated calcium chloride into 4 GPs
Group 2b: injection of nanoformulated calcium chloride into 2 GP
Group 3: injection of nanoformulated botox into 4 GPs

Paired comparisons are made for all autonomic nerve activity (ANA) quantitation and induction of AF. If the true difference in ANA between control and denervated is 16 µV-s, with a standard deviation of 10 µV-s, then we will need 5 animals to reject the null hypothesis that the means of LVEF (Left Ventricular Ejection Fraction) between denervation and non-denervation groups are equal, with probability of 0.8 and type 1 error of 0.05. This quantitative difference is based upon prior data (Tan et al Circulation 2008) of ANA difference between denervated and non-denervated, albeit the mode of denervation studied was different in the present study.

To account for fatalities associated with complications detailed in Appendix 5 from this protocol, there is an additional 20% requested (4 animals).

Group 1: injection of calcium chloride without nanoformulation = 5 animals
Group 2: injection of nanoformulated calcium chloride into 4 GPs= 5 animals
Group 2b: injection of nanoformulated calcium chloride into 2 GPs= 5 animals
Group 3: injection of nanoformulated botox into 4 GPs= 5 animals
20% to account for deaths= 4

TOTAL= 24

c. **Describe each procedure** to be performed on any animal on this protocol. (Use Appendix 9 to document any of these procedures that involve “departures” from the standards in the *Guide*. Consult the IACUC or the Attending Veterinarian for help in determining whether any “departures” are involved.)
►
<u>First surgery & Data Sciences International (DSI) nerve recording device implantation.</u>

The canine is pre-anesthetized with Acepromazine 0.05-0.1mg/kg approximately 1 hour before surgery. They are given Buprenorphine 0.01-0.02 mg/kg IM, Penicillin (900,000 units) IM and Famotidine 0.5-1 mg/kg PO prior to being anesthetized. They are anesthetized with Brevital 6-10 mg/kg IV (Pentobarbital 30mg/kg IV can be given if Brevital is unavailable) to effect to allow for intubation with a cuffed endotracheal tube. The endotracheal tube is then connected to a vaporizer and respirator for isoflurane induction and mechanical ventilation. Isoflurane 1-3 % mixed with oxygen is used for surgical plane of anesthesia throughout the surgery unless otherwise described. Heart rate, blood pressure and temperature will be recorded every 15 minutes. Average heart rate for the dogs under anesthesia is 85-100 bpm. If heart rate increases by more than 5bpm from the average to this point, isoflurane may be temporarily increase by .5% (no more than 3% during surgery). Each dog will be weighed before surgery to determine the maximum dose of anesthetic that is allowed to prevent them from overdose

Surgery will be performed under full aseptic technique. We will perform a left lateral thoracotomy incision at the T2-3 level. We will collect blood sample (less 10 mls) for analyses of norepinephrine levels from the aorta. We will implant a Data Sciences International (DSI) radiotelemetry device subcutaneously in canines. This device has three bipolar channels. We will implant one channel to record the left stellate ganglion nerve activity and one channel to record cardiac vagal nerve activity plus ECG. The third channel will be reserved for recording ganglionated plexi (GP) nerve activity. GP are located in epicardial fat pads. The electrode will be sutured onto the epicardial fat pad (but not onto atrial muscle underlying it). Occasionally, there is a risk of injuring the underlying atrial muscle resulting in bleeding. This bleeding will be quickly controlled and if necessary, sutured close with a non-cutting vicryl or silk suture. If this bleeding is not controllable, the animal will be euthanized by exsanguination under anesthesia.
In order to most accurately view autonomic nerve activity during an EP studies isoflurane may need to be turned off for a period of 30 minutes. We will first attempt to conduct the EP studies with isoflurane but if the nerve activity is altered due to this anesthetic, the following protocol will be followed. Pentobarbital will be administered as follows to keep the animal in a surgical plane of anesthesia. We will give 4-5mg/kg of pentobarbital initially and then turn off the isoflurane. The heart rate will be monitored closely. Average heart rate for the dogs under anesthesia is 85-100bpm. If heart rate increases by more than 5bpm from the average to this point, small doses of pentobarbital (2-3 mg/kg not to exceed a total 30 mg/kg including the loading dose). Once the nerve recording is finished, isoflurane will be resumed until end of surgery. Isoflurane has a nature of suppressing nerve activity and does so more than pentobarbital. For this reason, isoflurane is removed. If the maximum dose of pentobarbital is reached during surgery, isoflurane must be resumed and the EP study completed under the effects of pentobarbital. The DSI leads will be rechecked for stability, and the device will be implanted in a subcutaneous extrathoracic pocket. Once the device has been implanted in the pocket and the lead positions have been verified we will begin closing all surgical sites.

Some subjects will be transferred to protocols # 02289 a minimum of ten days following surgical thoracotomy for implantation of a neurophysiologic and electrophysiologic recording device. After 10 days animals will be evaluated for transfer to protocol #02289. The parameters assessed will include return of baseline function and activity; normal eating, sleeping and elimination behaviors; and minimal or no requirement for pain control medications, Additionally, the canine will need to have no weight loss for at least 3 days prior to transfer. Subjects will be returned to this protocol baring any physical impairments mentioned above, after the completion of procedures on protocol #02289.

EP study and AF induction. The purpose of the EPS is to determine the effective refractory period (ERP) of the atrium and inducibility of AF. A second purpose is to identify the GP by high frequency (20Hz) subthreshold stimulation. ERP and AF inducibility will be determined by programmed stimulation (single, double, triple premature stimuli) from three different sites in the LA (left atrial appendage,

posterior and anterior LA). The refractory period is the longest coupling interval of the premature beat that fails to capture the tissue. A short refractory period is a substrate for AF. Vulnerability to induced AF is assessed by sequential single, double and triple extrastimuli down to refractory period. Refractory period and vulnerability to AF are the primary end-points to be assessed across multiple surgeries, allowing comparison of the baseline state, vs a three-week postoperative state before and after administration of nanoformulated therapy to GP. High frequency subthreshold stimulation at 20Hz, at an output that does not capture atrium, is performed at the sites of GP. A positive response is defined by a change in heart rate of 5% or more after 10 sec of stimulation.
We will be using Bloom electrical stimulator. Programmed atrial stimulation will not result in comparable high ventricular rates because of AV node protection. The high frequency stimulation of GPs is a subthreshold stimulation meant to stimulate autonomic nerves, without stimulating the heart. In our experience (Tan et al Circulation 2009), we expect any AF to be induced will be non-sustained (lasting a matter of seconds to minutes only). If AF does not self terminate, we will perform burst overdrive pacing to terminate AF. In our experience, this is always successful. Thus, no animal will be left in AF at the end of the procedure. Each EPS typically lasts 30 minutes.

First the thoracotomy site will be closed with surgical steel wires to hold the ribs together. Then the overlying intercostal muscles and deep muscle layers will be closed with interrupted Vicryl sutures. Once this has been closed a previously implanted chest tube will be used to evacuate all air from the pleural cavity and reinflate the left lung. The skin will then be closed in two layers using running Vicryl suture lines. Finally reinforcing nylon vertical mattress sutures will be placed to hold the wound closed during the initial healing phase.

Animals are allowed to recover on the ventilator with 100% oxygen until swallowing reflex is noted. The incision will be sprayed with Vetericyn gel to promote wound healing. The endotracheal tube is removed and the canine is moved to a recovery cage with blankets and a warming pad. Once sternal, they receive meloxicam 0.2mg/kg IM.
The canine remains in the post operative recovery cage until the following morning when they are moved to the standard chain link run with padding and blankets. They are given buprenorphine 0.01-0.02 mg/kg IM twice a day for three days for analgesia. Carprofen (2mg/kg PO) can be given for 1-3 days if pain is noted after the buprenorphine regimen is completed. Famotidine 0.5-1.0 mg/kg PO or IV can be given as needed for nausea or appetite stimulant (metoclopramide 0.2-0.5 mg/kg IV or bismuth subsalicylate 262 mg PO, can be given if needed). Some canines are too active post operatively which prevents incision healing. In this case, they are given Diazepam 0.2-2mg/kg IM or PO twice a day as needed. Cefpodoxime 5mg/kg PO is given once a day for 10 days to prevent wound infection. If there is ongoing concern for wound infection while receiving Cefpodoxime (Baytril 5-20mg/kg PO can be given once a day for 10 days). IV Fluids are available with or without 5% dextrose if an animal has not resumed normal eating habits within 2 days. . Canine weights will be observed and recorded every other day while on antibiotics and then twice a weekly thereafter.

There is a very small risk of spontaneous ventricular fibrillation during the left thoracotomy surgery. Should this happen there are sterile internal defibrillator paddlers connected to a defibrillator set at 50 Joules prepared for resuscitation efforts. Epinephrine will also be administrated at a low dose (0.01 mg/kg) will be given every 3–5 min early in resuscitation efforts; a high dose (0.1 mg/kg) will be given after prolonged effort (15 minutes) with no response. Amiodarone (7mg/kg) will be used as an alternative. This is administrated in a single dose and repeated as needed every 5 minutes.

Autonomic Nerve Data Acquisition. Autonomic nerve recordings will be initiated 48 hours after surgery. This recording is done wirelessly without inhibiting the dogs' movement. We will record nerve activity for 1 week (baseline recording). The animal will have second surgery. After recovery from second surgery, the recordings will be repeated. .

Echocardiogram.
Baseline echocardiogram will be performed between first and second surgery (during the same period as autonomic nerve activity recording above), and post-GP injection echocardiogram the day before final surgery (6 days post-GP injection). We will use a GE-Echopac system.
This non-invasive procedure is not painful and should not cause any distress to the animal. However, it requires that the animal stands still and possibly lays supine for at least 10 minutes in order to obtain accurate cardiac images. Therefore, we believe that 2 different approaches will be required to obtain echocardiogram: 1) animal training with or without restraint, and/or 2) with a sedative.
Our first approach will be animal training to stand still and lay supine for 10 minutes to obtain echocardiogram. However, if the animal does not cooperate, we will have to perform echocardiogram under use of an oral sedative (Acepromazine 0.05-0.1mg/kg).

Exercise testing (ETT). A 9-minute exercise stress test will be performed at the same intervals as echocardiogram to evaluate peak heart rate, peak NA response to exercise and heart rate recovery. These studies will be performed once the animal is recovered from the prior surgery, typically at least 6 days after surgery.

To acclimate the animals to the DogPACER (canine specific treadmill) they will be initially introduced by letting them explore the exercise room and equipment until they have become comfortable in those surroundings. Presence of normal, relaxed behavior will signal that the dogs are ready for the next step, which is putting them on the treadmill while it's off. This will occur in small steps, putting them on for seconds and then extending the time. Each positive reaction will be rewarded with treats to encourage the dogs' learning process. When the dogs have become relaxed with the task of being on the still treadmill, they will next be put on the treadmill at its slowest speed, 0.5 mph. Two people will assist in this process; one person will hold the leash of the dog and stand in front of the treadmill offering rewards for positive behavior while the other will stand behind the animal making sure that she does not slide off of the machine, jump off of the sides and also to help the dog move their feet until she begins to understand and be comfortable with the movement herself.
The treadmill workout will be done a total of 2 times in our study. The first (baseline) workout will be performed at least 6 days post the first surgery after sutures have been removed. The second and final work out will occur 6 days post the second surgery after sutures have been removed. Each workout lasts 10 minutes, in which the dogs will complete 3 stages, each lasting 3 minutes. The first setting will be at 1.1 mph followed by three minutes at 2.3 mph and finally three minutes at 3.3 mph. A canine's top speed is 20-30mph so the top speed of this test has the dog at a fast walk to slow jog.

Heart rate will be recorded before, at the peak of workout and at the finish. At that point, heart rate will be recorded every minute until it returns to baseline. The amount of time it takes for heart rate to return to baseline post-workout is the true measure of fitness. Heart rate can be displayed and monitored during the workout by a pacing analyzer that connects wirelessly to pacemaker implanted. In this way, we can also assess arrhythmias as the mild cardiomyopathy develops. Blood samples will be obtained through an IV catheter placed in the jugular vein or brachial vein. Blood will be drawn 4 times: once before, during each of 3 treadmill stages and recovery phase without exceeding 15mL (less than 1% of animal's body weigh). The blood will be drawn up through a syringe connected to a sterile intravenous catheter.

Intravenous pharmacological challenge. In order to understand and validate recordings in the autonomic nerves and relationship to heart rhythm and blood pressure, we plan to pharmacologically

stimulate autonomic nerves by administration of short-acting intravenous vasoactive drugs (clonidine, and phenylephrine) during the chronic monitoring phase.

These drugs used are ones commonly used in clinical practice in humans but have been also used in canines. They are all short-acting drugs whose half-lives do not exceed 12 hours when given orally. Therefore, when administered IV, their effects peak within minutes and half-lives usually less than 4 hours as described below:

1. IV clonidine (10 µg/kg) is an alfa-2 agonist which will suppress central sympathetic nerve discharge by acting on imidazoline receptors in the midbrain, resulting in slow heart rate. It will initially cause a rise in blood pressure through action on peripheral alfa 2 receptors in the postsynaptic terminal resulting in vasoconstriction. Subsequently, it will lower blood pressure by acting on peripheral pre-synaptic alfa-2 receptors in sympathetic nerve terminals and by suppressing central sympathetic output. IV Clonidine peaks within an hour and has a plasma half-life of 2-3 hours. Blood pressure and heart rate are expected to drop but the doses used have been reported in the literature [Cavero, Br. J Pharmacol 1980; 70:269]. We expect that the effects are transient and will not have long term sequelae.
   There is a slight risk that ventricular tachycardia could occur. This has been observed to happen in denervated animals in another protocol, however this is not a problem that is anticipated in these animals. To combat this, the animal will be monitored for 60 minutes post administration. If sustained VT is observed on the DSI recording for more than 2 minutes, the animal will be administered Amiodarone (7mg/kg IV, every 5 minutes as needed). If hypotension occurs, we will administer fluids. Because this medication is transient, we do not expect sudden cardiac death to occur. In other protocols, we have not observed any evidence of dramatic hypotension as a result of this medication.
2. IV phenylephrine 0.01mg/kg. Phenylephrine is a vasopressor used to increase BP. This will be given as an IV bolus. The increase in BP will suppress sympathetic nerve activity and potentiate vagal nerve activity. We expect that the effects are transient and will not have long term sequelae. [Varma S, Circulation Research 1960;8:1182]. [Moise, N., Moon, P. F., Flahive, W. J., Brittain, D., Pride, H., Lewis, B. A., ... & Gilmour, R. F. (1996). Phenylephrine-Induced Ventricular Arrhythmias in Dogs with Inherited Sudden Death. *Journal of cardiovascular electrophysiology*, *7*(3), 217-230.]

We will maintain a log of blood pressure readings, heart rate, time of administration and physical characteristics during the monitoring phase and keep this information in the animals folder for review. We continuously monitor BP and heart rate via DSI recordings for 1 hour. The medications peak rapidly (within a few minutes), and its effects wane after about 20 minutes. By 1 hour, the vital signs are back to baseline.

Second surgery. This will be at least 21 days after first surgery. The canine is pre-anesthetized with Acepromazine 0.05-0.1mg/kg approximately 1 hour before surgery. They are given Buprenorphine 0.01-0.02 mg/kg IM, Penicillin (900,000 units) IM and Famotidine 0.5-1 mg/kg PO prior to being anesthetized. They are anesthetized with Brevital 6-10 mg/kg IV (Pentobarbital 30mg/kg IV can be given if Brevital is unavailable) to effect to allow for intubation with a cuffed endotracheal tube. The endotracheal tube is then connected to a vaporizer and respirator for isoflurane induction and mechanical ventilation. Isoflurane 1-3 % mixed with oxygen is used for surgical plane of anesthesia throughout the surgery unless otherwise described. Heart rate, blood pressure and temperature will be recorded every 15 minutes. Average heart rate for the dogs under anesthesia is 85-100 bpm. If heart rate increases by more than 5bpm from the average to this point, isoflurane may be temporarily increase by .5% (no more than 3% during surgery). Each dog will be weighed before surgery to determine the maximum dose of anesthetic that is allowed to prevent them from overdose

Second surgery will consist of either (a) left thoracotomy only to target left sided ganglionated plexi for therapy, or (b) right thoracotomy only to target right sided ganglionated plexi, or (c) bilateral right and left thoracotomy at 5th intercostal spaces, in order to target all 4 ganglionated plexi. Bilateral thoracotomy is needed due to the need to limit the incision size in a survival surgery. This limits access to only unilateral ganglionated plexi. Our data so far demonstrates a cumulative effect of treating more ganglionated plexi, We also expect that in humans, all 4 GP will be targeted during heart surgery as the approach of median sternotomy allows easy access to all. Therefore, treating all 4 GP is the most clinically relevant scenario and one we aim to test scientifically. However, in survival animal model, we will avoid median sternotomy due to long recovery times and instead rely on bilateral limited thoracotomies to access and treat all 4 GP. Note that survival bilateral survival thoracotomy has been reported before (Tan AY, Zhou S, Ogawa M, Song J, Chu M, Li H, Fishbein MC, Lin S-F, Chen LS, Chen P-S. Neural Mechanisms of Paroxysmal Atrial Fibrillation and Paroxysmal Atrial Tachycardia in Ambulatory Canines. Circulation 2008; 118(9):916-25. PMID: 18697820.). For the left thoracotomy and bilateral thoracotomy group, we will start with a limited left thoracotomy. This will be one or two interspaces lower than the one performed during first surgery. The original incision in the 3rd or 4th intercostal space will not be reaccessed to limit the risk of bleeding, and to avoid areas with the densest adhesions. Any adhesions will be carefully released to avoid injury to the lungs and blood vessels. We expect to be able to limit the incision size of the present incision to about 4 cm, which will speed healing. This limited incision will allow us to directly visualize the GPs on the left side, inject them with nanoparticles, and subsequently induce AF by programmed electrical stimulation. The technique of injection is to simply use a 1cc syringe attached to an insulin needle (smallest possible gauge). We will make a 45 degree bend to the needle shaft so as to be able to inject in a horizontal plane into the epicardial fat pad (parallel to the epicardial surface), instead of vertically to avoid penetrating into the atrial muscle underneath the fat. In terms of the EP study, this method is well known to induce AF experimentally and clinically, and will not result in rapid heart rates as the atria, not the ventricle, is paced. Following this, the left thoracotomy will be closed.

In bilateral thoracotomy group,,the dog will be flipped to the other side and re-prepped for aseptic surgery. For these animals and the ones having a right thoracotomy only, we will begin with a right thoracotomy in the 5th intercostal space. Again, a limited thoracotomy will be performed as per left side. This limited incision (<5cm) will allow us to directly visualize the GPs on the right side, inject them with nanoparticles, and subsequently induce AF by programmed electrical stimulation.

In the case of a right thoracotomy after a left thoracotomy, we will not experience any adhesions as the side is far away from the initial left thoracotomy. This allows us to visualize the right sided GP with a minimal incision. The technique of injection of GP is to simply use a 1cc syringe attached to an insulin needle (smallest possible gauge). We will make a 45 degree bend to the needle shaft so as to be able to inject in a horizontal plane into the epicardial fat pad (parallel to the epicardial surface), instead of vertically to avoid penetrating into the atrial muscle underneath the fat. In terms of the EP study, this method is well known to induce AF experimentally and clinically, and will not result in rapid heart rates as the atria, not the ventricle, is paced. In Group 1 (N=5), 4 GPs will be injected with calcium chloride alone (without nanoparticles). In Group 2a (N=5), all 4 GPs will be injected with nano-formulated calcium chloride. In Group 2b (N=5), two left or right sided GPs (not both) will be injected with either nano-formulated calcium chloride.. In Group 3 (N=5), all 4 GPs will be injected with nano-formulated calcium chloride.

AF induction will be performed before AND 1 hour after GP injection. AF induction before GP injection will serve as internal negative control (with the animal being a postoperative state after first surgery).

In order to most accurately view autonomic nerve activity during an EP studies isoflurane may need to be turned off for a period of 30 minutes. We will first attempt to conduct the EP studies with isoflurane

but if the nerve activity is altered due to this anesthetic, the following protocol will be followed. Pentobarbital will be administered as follows to keep the animal in a surgical plane of anesthesia. We will give 4-5mg/kg of pentobarbital initially and then turn off the isoflurane. The heart rate will be monitored closely. Average heart rate for the dogs under anesthesia is 85-100bpm. If heart rate increases by more than 5bpm from the average to this point, small doses of pentobarbital (2-3 mg/kg not to exceed a total 30 mg/kg including the loading dose). Once the nerve recording is finished, isoflurane will be resumed until end of surgery. Isoflurane has a nature of suppressing nerve activity and does so more than pentobarbital. For this reason, isoflurane is removed. If the maximum dose of pentobarbital is reached during surgery, isoflurane must be resumed and the EP study completed under the effects of pentobarbital. \

EP study and AF induction. The purpose of the EPS is to determine the effective refractory period (ERP) of the atrium and inducibility of AF. A second purpose is to identify the GP by high frequency (20Hz) subthreshold stimulation. ERP and AF inducibility will be determined by programmed stimulation (single, double, triple premature stimuli) from three different sites in the LA (left atrial appendage, posterior and anterior LA). The refractory period is the longest coupling interval of the premature beat that fails to capture the tissue. A short refractory period is a substrate for AF. Vulnerability to induced AF is assessed by sequential single, double and triple extrastimuli down to refractory period. Refractory period and vulnerability to AF are the primary end-points to be assessed across multiple surgeries, allowing comparison of the baseline state, vs a three-week postoperative state before and after administration of nanoformulated therapy to GP. High frequency subthreshold stimulation at 20Hz, at an output that does not capture atrium, is performed at the sites of GP. A positive response is defined by a change in heart rate of 5% or more after 10 sec of stimulation. We will be using Bloom electrical stimulator. Programmed atrial stimulation will not result in comparable high ventricular rates because of AV node protection. The high frequency stimulation of GPs is a subthreshold stimulation meant to stimulate autonomic nerves, without stimulating the heart. In our experience (Tan et al Circulation 2009), we expect any AF to be induced will be non-sustained (lasting a matter of seconds to minutes only). If AF does not self terminate, we will perform burst overdrive pacing to terminate AF. In our experience, this is always successful. Thus, no animal will be left in AF at the end of the procedure. Each EPS typically lasts 30 minutes.
Animals are allowed to recover on the ventilator with 100% oxygen until swallowing reflex is noted. The incision will be sprayed with Vetericyn gel to promote wound healing. The endotracheal tube is removed and the canine is moved to a recovery cage with blankets and a warming pad. Once sternal, they receive meloxicam 0.2mg/kg IM.

The canine remains in the post operative recovery cage until the following morning when they are moved to the standard chain link run with padding and blankets. They are given buprenorphine 0.01-0.02 mg/kg IM twice a day for three days for analgesia. Carprofen (2mg/kg PO) can be given for 1-3 days if pain is noted after the buprenorphine regimen is completed. Famotidine 0.5-1.0 mg/kg PO or IV can be given as needed for nausea or appetite stimulant (metoclopramide 0.2-0.5 mg/kg IV or bismuth subsalicylate 262 mg PO, can be given if needed). Some canines are too active post operatively which prevents incision healing. In this case, they are given Diazepam 0.2-2mg/kg IM or PO twice a day as needed. Cefpodoxime 5mg/kg PO is given once a day for 10 days to prevent wound infection. If there is ongoing concern for wound infection while receiving Cefpodoxime (Baytril 5-20mg/kg PO can be given once a day for 10 days). IV Fluids are available with or without 5% dextrose if an animal has not resumed normal eating habits within 2 days. . Canine weights will be observed and recorded every other day while on antibiotics and then twice a weekly thereafter.

There is a very small risk of spontaneous ventricular fibrillation during the left thoracotomy surgery.

Should this happen there are sterile internal defibrillator paddlers connected to a defibrillator set at 50 Joules prepared for resuscitation efforts. Epinephrine will also be administrated at a low dose (0.01 mg/kg) will be given every 3–5 min early in resuscitation efforts; a high dose (0.1 mg/kg) will be given after prolonged effort (15 minutes) with no response. Amiodarone (7mg/kg) will be used as an alternative. This is administrated in a single dose and repeated as needed every 5 minutes.

Final surgery. This will be 7 days after second surgery. The canine is pre-anesthetized with Acepromazine 0.05-0.1mg/kg approximately 1 hour before surgery. They are anesthetized with Brevital 6-10 mg/kg IV (Pentobarbital 30mg/kg IV can be given if Brevital is unavailable) to effect to allow for intubation with a cuffed endotracheal tube. The endotracheal tube is then connected to a vaporizer and respirator for isoflurane induction and mechanical ventilation. Isoflurane 1-3 % mixed with oxygen is used for surgical plane of anesthesia throughout the surgery unless otherwise described. Heart rate, blood pressure and temperature will be recorded every 15 minutes. Average heart rate for the dogs under anesthesia is 85-100 bpm. If heart rate increases by more than 5bpm from the average to this point, isoflurane may be temporarily increase by .5% (no more than 3% during surgery). Each dog will be weighed before surgery to determine the maximum dose of anesthetic that is allowed to prevent them from overdose. Then a left thoracotomy will be performed under isoflurane anesthesia. Blood will be collected (less than 10 mls)for norepinephrine analyses from aorta. Repeat EP study and AF induction will be re-attempted as explained above for first and second surgery. During this time, pentobarbital will be administered as follows to keep the animal in a surgical plane of anesthesia. We will give 4-5mg/kg of pentobarbital initially and then turn off the isoflurane. The heart rate will be monitored closely. Average heart rate for the dogs under anesthesia is 85-100bpm. If heart rate increases by more than 5bpm from the average to this point, small doses of pentobarbital (2-3 mg/kg not to exceed a total 30 mg/kg including the loading dose) will be administered to effect. Once the nerve recording is finished, isoflurane will be resumed until end of surgery. Isoflurane has a nature of suppressing nerve activity and does so more than pentobarbital. For this reason, isoflurane is removed. If the maximum dose of pentobarbital is reached during surgery, isoflurane must be resumed and the EP study completed under the effects of pentobarbital. Towards the end of surgery, whilst under isoflurane, the animal is turned over and a right thoracotomy performed in order to test AF induction from the right side (stimulation using the same programmed stimulation protocol as the left side, but from the right atrial appendage). After this, the animal will be euthanized by exsanguination under isoflurane anesthesia. The tissues stored in 4% formalin for histological analyses.

Species. Justify the choice of species for this protocol.

► Canines have very similar physiology to humans. In addition, there are significant differences in cardiac physiology between small animal species and humans. The experimental techniques, electronic pacemakers and leads available are large and require a larger species.. The radiotelemetry device is large and will require internal implantation and observation for several weeks. Additionally, dogs have a His-Purkinje system located in endocardium, very similar to the human's heart, which pigs and other larger animals do not have

Personnel

D. **Current qualifications and training.** (For personnel who require further training, plans for additional training will be requested in Item F.)

1. PI
   Name► [redacted]
   Animal research experience ► [redacted]

[redacted]

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
First surgery & Data Sciences International (DSI) nerve recording device implantation	[redacted]
Echocardiogram	[redacted]
Second Surgery (Injection of Nanoparticles)	[redacted]

2. Other research personnel (copy the lines below for each individual)

Name► [redacted]
Animal research experience ► ► [redacted]

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
First surgery	[redacted]

	[redacted].
Second surgery	[redacted].
Final surgery	[redacted].

Name [redacted]
Animal research experience ►[redacted]

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
First surgery & Data Sciences International (DSI) nerve recording device implantation	[redacted]
Echocardiogram	[redacted]
Exercise testing (ETT)	[redacted]
Second Surgery (Nanoparticle Injection)	[redacted].
Final Surgery	[redacted]

Name► [redacted]

Animal research experience ► [redacted]

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
First surgery & Data Sciences International (DSI) nerve recording device implantation	[redacted]
Echocardiogram	[redacted]
Exercise testing (ETT)	[redacted]
Second Surgery (Nanoparticle Injection)	[redacted]
Final Surgery	[redacted]

3. VMU animal care and veterinary support staff personnel (copy the lines below for each individual)

Name►
Qualifications to perform specific support procedures in the animals on this protocol

Specific support procedure(s) assigned to this individual	Qualifications for performing each support procedure in the species described in this ACORP (e.g., AALAS certification, experience, or completion of special training)

4. For each of the research personnel listed in items 1 and 2 above, enter the most recent completion date for each course

Name of Individual	Working with the VA IACUC	ORD web-based species specific course (Identify the species)	Any other training required locally (Identify the training)

[redacted]	[redacted]	[redacted]	
[redacted]	[redacted]	[redacted]	
[redacted]	[redacted]	[redacted]	
[redacted]	[redacted]	[redacted]	

F. **Training to be provided.** List here each procedure in Item E for which anyone is shown as "to be trained", and describe the training. For each procedure, describe the type of training to be provided, and give the name(s), qualifications, and training experience of the person(s) who will provide it. If no further training is required for anyone listed in Item E, enter "N/A"
►

First surgery & Data Sciences International (DSI) nerve recording device implantation: [redacted]
Exercise testing (ETT): [redacted]
Second Surgery (Nanoparticle Injection): [redacted]
AF Induction: AF will be induced by methods described previously. Briefly, high frequency stimulation of GPs along with programmed atrial stimulation will be used to induce AF, using Bloom electrical stimulator. Programmed stimulation involves burst pacing as well as premature stimulus at twice the capture threshold, as utilized in standard clinical electrophysiology. Programmed atrial stimulation will not result in comparable high ventricular rates because of AV node protection. The high frequency stimulation of GPs is a subthreshold stimulation meant to stimulate autonomic nerves, without stimulating the heart. In our experience [redacted] we expect any AF to be induced will be non-sustained (lasting a matter of seconds to minutes only).
Final Surgery: [redacted]

G. **Occupational Health and Safety.**

1. Complete one line in the table below for each of the personnel identified in Item E:

Name	Enrollment in OHSP		Declined optional services	Current on Interactions with OHSP? (yes/no)
	VA program	Equivalent Alternate Program – identify the program		
[redacted]	(X)	( )	( )	Yes
[redacted]	(X)	( )	( )	Yes
[redacted]	(X)	( )	( )	Yes
[redacted]	(X)	( )	( )	Yes
	(X)	( )	( )	Yes
[redacted]	(X)	( )	( )	Yes

2. Are there any non-routine OHSP measures that would potentially benefit, or are otherwise required for, personnel participating in or supporting this protocol?

► ( ) Yes. Describe them ►

► (X) No.

Animals Requested

H. **Animals to be Used.** Complete the following table, listing the animals on separate lines according to any specific features that are required for the study (see ACORP Instructions, for guidance, including specific terminology recommended for the "Health Status" column):

Description (include the species and any other special features not shown elsewhere in this table)	Gender	Age/Size on Receipt	Source (e.g., Name of Vendor, Collaborator, or PI of local breeding colony)	Health Status
Mongrel Canines	Female	6-12 months/ 25-30 kg	[redacted]	Conditioned

I. **Numbers of animals requested.** See ACORP Instructions, for descriptions of the categories and how to itemize the groups of animals.

USDA Category B

Procedures►						
Species / Experimental Group / Procedures(s)	Year 1	Year 2	Year 3	Year 4	Year 5	**Category B TOTAL**

USDA Category C

Procedures►						
Species / Experimental Group / Procedure(s)	Year 1	Year 2	Year 3	Year 4	Year 5	**Category C TOTAL**

USDA Category D

Procedures► First surgery & Data Sciences International (DSI) nerve recording device implantation, Second Survival Surgery, AF induction						
Species / Experimental Group / Procedure(s)	Year 1	Year 2	Year 3	Year 4	Year 5	**Category D TOTAL**
Group 1: CaCl2 only control		5				5
Group 2a: Nanoformulated CaCl2 in 4 GPS		6				6
Group 2.b Nanoformulated CaCl2 in 2 GPS		6				6
Group 3: Nanoformulated botox in 4 GPS	7					7

USDA Category E

Procedures►						
Species / Experimental Group / Procedure(s)	Year 1	Year 2	Year 3	Year 4	Year 5	**Category E TOTAL**

TOTALS over all Categories

Species / Experimental Group /Procedure(s)	Year 1	Year 2	Year 3	Year 4	Year 5	**GRAND TOTAL**
Groups 1, 2.a,2.b, 3	7	17				24

J. **Management of USDA Category D procedures**. Indicate which statement below applies, and provide the information requested.

► ( ) This protocol does NOT include any Category D procedures.

► (X) This protocol INCLUDES Category D procedures. List each Category D procedure and provide the information requested. (For surgical procedures described in Appendix 5, only identify the procedure(s) and enter "See Appendix 5 for details.)

Procedure	Monitoring (indicate the method(s) to be used, and the frequency and duration of monitoring through post-procedure recovery)	Person(s) responsible for the monitoring	Method(s) by which pain or distress will be alleviated during or after the procedure (include the dose, route, and duration of effect of any agents to be administered)
First surgery & Data Sciences International (DSI) nerve recording device implantation	See Appendix 5	[redacted]	See Appendix 5
Second Survival Surgery (Nanoparticle Injection).	See Appendix 5	[redacted]	See Appendix 5
AF induction	See Appendix 5	[redacted]	See Appendix 5

K. **Justification of Category E procedures.** Indicate which statement below applies, and provide the information requested.

► (X) This protocol does NOT include any Category E procedures

► ( ) This protocol INCLUDES Category E procedures. Identify each Category E procedure included in this ACORP and justify scientifically why the pain or distress cannot be relieved.
►

Veterinary Care and Husbandry

L. **Veterinary Support**.

1. Identify the laboratory animal veterinarian who is responsible for ensuring that the animals on this protocol receive appropriate veterinary medical care.

Name► [redacted]
Institutional affiliation► [redacted]
email contact► [redacted]

2. Veterinary consultation during the planning of this protocol.

   Name of the laboratory animal veterinarian consulted► [redacted]
   Date of the veterinary consultation (meeting date, or date of written comments provided by the veterinarian to the PI) ►[redacted]

M. **Husbandry.** As a reference <u>for the animal husbandry staff</u>, summarize here the husbandry requirements of the animals on this protocol. (Use Appendix 6 to justify the use of any special husbandry and to detail its effects on the animals. Use Appendix 9 to document any aspects of the husbandry that involve "departures" from the standards in the *Guide*. Consult the IACUC or the Attending Veterinarian for help in determining whether any "departures" are involved.)

1. Caging needs. Complete the table below to describe the housing that will have to be accommodated by the housing sites for this protocol:

a. Species	b. Type of housing*	c. Number of individuals per housing unit**	d. Is this housing consistent with the *Guide* and USDA regulations? (yes/no***)	e. Estimated maximum number of housing units needed at any one time
Canines	**Chain link run, 3x6 feet cage**	1	No	5

*See ACORP Instructions, for guidance on describing the type of housing needed. If animals are to be housed according to a local Standard Operating Procedure (SOP), enter "standard (see SOP)" here, and enter the SOP into the table in Item Y. If the local standard housing is not described in a SOP, enter "standard, see below" in the table and describe the standard housing here:
► **Chain link run, 3x6 feet cages**

** The *Guide* states that social animals should generally be housed in stable pairs or groups. Provide a justification if any animals will be housed singly (if species is not considered "social", then so note)
► **Dogs are housed singly in chain link runs but can socialize with one another since each room has two to five dog runs. In addition, while their runs are being cleaned on a daily basis, pairs of dogs are allowed to exercise and play together in a designated "romper room". Animals are fitted with DSI transmitters and need to be housed singly in a cage for which DSI receivers are installed to receive signals from the transmitters. Mixing dogs will result in data cross talk.**

***Use Appendix 9 to document "departures" from the standards in the *Guide*.

2. Enrichment. Complete the table below to indicate whether "standard" exercise and environmental enrichment will be provided to the animals on this protocol, or whether any special supplements or restrictions will be required (See ACORP Instructions, for more information on enrichment

requirements. Use Appendix 9 to document any enrichments requirements that represent "departures" from the standards in the *Guide*.):

a. Species	b. Description of Enrichment*	c. Frequency
Canines	10 minute exercise regimen daily, standard, see below	Standard, see below

*If enrichment will be provided according to a local SOP, enter "standard (see SOP)" and enter the SOP into the table in Item Y. If the local standard enrichment is not described in a SOP, enter "standard, see below", and describe the standard species-specific enrichment here.
► **Per SOP, dogs can see, smell and interact with each other through the chain link. They are provided 10-20 minutes of interaction with the animal caretaker daily and toys/treats are provided and rotated weekly**

3. Customized routine husbandry. Check all of the statements below that apply to the animals on this protocol, and provide instructions to the animal husbandry staff with regard to any customized routine husbandry needed.

► ( ) This ACORP INCLUDES genetically modified animals.

List each group of genetically modified animals, and describe for each any expected characteristic clinical signs or abnormal behavior related to the genotype and any customized routine husbandry required to address these. For genetic modifications that will be newly generated on or for this protocol, describe any special attention needed during routine husbandry to monitor for unexpected clinical signs or abnormal behavior that may require customized routine husbandry.
►

► ( ) Devices that extend chronically through the skin WILL be implanted into some or all animals on this protocol. Describe any customized routine husbandry to be provided by animal husbandry staff to minimize the chances of chronic infection where the device(s) penetrate the skin.
►

► (X) Some or all of the animals on this protocol WILL require other customized routine husbandry by the animal husbandry staff, beyond what has been described above. Describe the special husbandry needed.
► **This pertains to cage cleaning and letting the animal out of the cage. To avoid cross talk between different transmitters being picked up by a receiver, the dogs should only be let out one at a time, and at a pre-specified time (so it is clear what period of time data will be lost or there is potential cross talk).**

► ( ) This ACORP does NOT include use of any animals that will require customized routine husbandry.

N. **Housing Sites**. Document in the tables below each location where animals on this protocol may be housed.

► (X) Housing on VA property. Identify each location on VA property where animals on this protocol will be housed, and indicate whether or not each location is inside the VMU.

Building	Room number	Inside of VMU?	
		Yes	No
[redacted]	[redacted]	(X)	( )
		( )	( )
		( )	( )

► ( ) Housing in non-VA facilities. Identify each location not on VA property where animals on this protocol will be housed, and provide the information requested in the table.

Name of Non-VA Facility	Is this facility accredited by AAALAC?		Building	Room Number
	Yes -- enter status*	No**		
	( )	( )**		
	( )	( )**		
	( )	( )**		

*See ACORP Instructions, for a list of AAALAC accreditation status options.

**For any facility listed above that is not accredited by AAALAC, attach documentation that a waiver has been granted by the CRADO.

Special Features

O. **Antibody Production.** Will any of animals on this protocol be used for the production of antibodies?

► ( ) Some or all of the animals on this protocol WILL be used in the production and harvesting of antibodies. Check “Appendix 2” in Item Y, below, and complete and attach Appendix 2, “Antibody Production”.

► (X) NO animals on this protocol will be used in the production and harvesting of antibodies.

P. **Biosafety.** Will any substances (other than those used in routine husbandry or veterinary care) be administered to the animals on this protocol?

► (X) This protocol INVOLVES administration of substances to the animals other than those used in routine husbandry and veterinary care. Check “Appendix 3” in Item Y, below, and complete and attach Appendix 3, “Biosafety”.

► ( ) This protocol does NOT involve administration of any substances to the animals other than those used in routine husbandry and veterinary care.

Q. **Locations of procedures.** Complete the table below, listing the location(s), inside or outside of the animal facility, for each of the procedures to be performed on animals on this protocol.

Procedure	Surgical?		Bldg/Room Number	Requires transport through non-research areas?	
	Yes	No		Yes – describe method of discreet transport	No
First surgery & Data Sciences International (DSI) nerve recording device implantation	(X)	( )	BC 158	( )	(X)
Second Survival Surgery (Nanoinjection)	(X)	( )	BC 158	( )	(X)
ETT (Treadmill)	( )	(X)	BC-108	( )	(X)
Echocardiogram	( )	(X)	3D-108	(X) transported by dedicated elevator	( )
Blood Draw	( )	(X)	BC108	( )	(X)
Nerve Recording	( )	(X)	BC 121&124	( )	(X)
Final Surgery	(X)	( )	BC 158	( )	(X)

R. **Body Fluid, Tissue, and Device Collection.** List each body fluid, tissue, or device to be collected, and complete the table below to indicate the nature of the collection. Check the relevant Appendices in Item Y, below, and complete and attach them, as shown in the column headings.

Body Fluid, Tissue, or Device to be Collected	Collected AFTER Euthanasia	Collected BEFORE Euthanasia		
		Blood Collection Associated with Antibody Production (Appendix 2, "Antibody Production")	Collected as Part of a Surgical Procedure (Appendix 5, "Surgery")	Other Collection from Live Animals (Appendix 4, "Antemortem Specimen Collection")
Blood	**( )**	**( )**	**( )**	**(X)**
Heart	**(X)**	**( )**	**( )**	**( )**
DSI Device	**(X)**	**( )**	**( )**	**( )**

S. **Surgery.** Does this protocol include any surgical procedure(s)?

► (X) Surgery WILL BE PERFORMED on some or all animals on this protocol. Check "Appendix 5" in Item Y, below, and complete and attach Appendix 5, "Surgery".

► ( ) NO animals on this protocol will undergo surgery.

T. **Endpoint criteria.** Describe the criteria that will be used to determine when animals will be removed from the protocol or euthanatized to prevent suffering. (Use Appendix 9 to document any "departures" from the standards in the *Guide* represented by these criteria. Consult the IACUC or the Attending Veterinarian for help in determining whether any "departures" are involved.)
► If animals suffer from heart failure, they will satisfy end point and euthanasia will be performed. increased heart rate/anxiety. Signs include weight loss of more than 12%, not eating, lethargy, weight gain due to fluid retention, signs of fluid accumulation in prone locations (edema), in the case of canines, in the abdomen (ascites). Weights will be monitored twice a week starting after the first surgical procedure and continued until the animal completes the protocol. If weights drop more than 5%, the veterinarian will be notified and weights will be measured daily until completion of protocol or until weight returns to normal. This weight will be logged in the animals file. .Blood pressure will also be monitored weekly and recorded in the chart. Alternatively, if they were to suffer a serious postoperative complication such as uncontrollable bleeding, including bleeding from the pulmonary vascular tree, pneumothorax, hemothorax, end point criteria will be reached. If there is uncontrollable infection of the pocket in which DSI is placed, and these cannot be controlled by antibiotics, then end point criteria is reached. Pocket infection presents as pocket swelling with purulence, erythema, pain, fever, anorexia, which does not resolve with antibiotics. Note that pocket seroma (a pooling of non-infectious fluid in the device pocket) is common after surgery but this presents merely as pocket swelling without inflammatory features as above. If the animal experiences nausea and anorexia and weight loss greater than 12% due to vagal injury (from electrode implanted into vagus nerve), the animal will be euthanized.

U. **Termination or removal from the protocol**. Complete each of the following that applies:

► ( ) Some or all animals will NOT be euthanatized on this protocol. Describe the disposition of these animals. (Use Appendix 9 to document any "departures" from the standards in the *Guide* represented by these methods of disposition. Consult the IACUC or the Attending Veterinarian for help in determining whether any "departures" are involved.)
►

► (X) Some or all animals MAY be euthanatized as part of the planned studies. Complete the table below to describe the exact method(s) of euthanasia to be used. (Use Appendix 9 to document any departures from the standards in the *Guide* represented by these methods. Consult the IACUC or the Attending Veterinarian for help in determining whether any "departures" are involved.)

Check each method that may be used on this protocol	Method of Euthanasia	Species	AVMA Classification		
			Acceptable	Conditionally Acceptable	Unacceptable

( )	CO_2 from a compressed gas tank Duration of exposure after apparent clinical death► Method for verifying death► Secondary physical method►		( )	( )	( )
(X)	Anesthetic overdose Agent► Pentobarbital Dose►100mg/kg Route of administration► IV	Canine	(X)	( )	( )
( )	Decapitation under anesthesia Agent► Dose► Route of administration►		( )	( )	( )
( )	Exsanguination under anesthesia Agent► Isoflurane Dose►1-3% Route of administration► inhaled	Canine	(X)	( )	( )
( )	Other (Describe) ►		( )	( )	( )
( )	Other (Describe) ►		( )	( )	( )

1. For each of the methods above that is designated as "Conditionally Acceptable" by the AVMA, describe how the conditions for acceptability will be met:
   ►

2. For each of the methods above that is designated as "Unacceptable" by the AVMA, give the scientific reason(s) that justify this deviation from the AVMA Guidelines:
   ►

3. Identify all research personnel who will perform euthanasia on animals on this protocol and describe their training and experience with the methods of euthanasia they are to use in the species indicated.
   ► [redacted] **will perform euthanasia.** [redacted]**, and** [redacted] **will assist.** [redacted]

[redacted] **has extensive experience in canine surgery of the nature described for terminal surgery.**

4. Instructions for the animal care staff in case an animal is found dead.
   a. Describe the disposition of the carcass, including any special safety instructions. If disposition is to be handled according to a local SOP, enter "according to local SOP" and enter the information requested about the SOP into the table in Item Y.
   ► [redacted] **is to be informed immediately. The carcass should be kept in a refrigerator. A postmortem examination will be performed and the hearts harvested for histopathology and storage.**

   b. Describe how the PI's staff should be contacted.
   ► ( ) Please contact a member of the PI's staff immediately. (Copy the lines below for each individual who may be contacted)
   Name► [redacted]
   Contact Information► **(c)** [redacted] **; email:** [redacted]

   ► ( ) There is no need to contact the PI's staff immediately. Describe the routine notification procedures that will be followed. If the routine notification procedures are described in a local SOP, enter "according to local SOP" and enter the information requested about the SOP into the table in Item Y.
   ►

V. **Special Procedures.** List each special procedure (including special husbandry and other special procedures) that is a part of this protocol, and specify where the details of the procedure are documented. See ACORP Instructions, for examples.

Name of Procedure	Identify Where the Details of the Procedure are Documented		
	SOP (title or ID number)*	Other Items in this ACORP -- specify the Item letter(s)	Appendix 6
Nerve Recording		Items: C.2.c	(X)**
Treadmill Exercise		Items: C.2.c	()**
Echocardiogram		Items: C.2.c	( )**
Electrocardiogram		Items: C.2.c	( )**
Blood Draw		Items C.2.c	

*If any special procedure is detailed in a SOP, identify the SOP and enter the information requested about the SOP in the table in Item Y.

**If any special procedure is detailed in Appendix 6, check "Appendix 6" in Item Y, below, and complete and attach Appendix 6.

(Use Appendix 9 to document any "departures" from the standards in the *Guide* represented by these procedures. Consult the IACUC or the Attending Veterinarian for help in determining whether any "departures" are involved.)

W. **Consideration of Alternatives and Prevention of Unnecessary Duplication.** These are important to minimizing the harm/benefit to be derived from the work.

1. Document the database searches conducted.
   List each of the potentially painful or distressing procedures included in this protocol.
   ► Thoracotomy, Second thoracotomy, blood draws, and AF Induction.

   Then complete the table below to document how the database search(es) you conduct to answer Items W.2 through W.5 below address(es) each of the potentially painful or distressing procedures.

Name of the database	Date of search	Period of years covered by the search	Potentially painful or distressing procedures addressed	Key words and/or search strategy used	Indicate which mandate each search addressed			
NIH PubMed	6/27/2016	2006-2016	First surgery, Second Surgery, Atrial fibrillation induction, nanoparticle injection	atrial fibrillation, arrhythmia, nanoparticle injection survival second thoracotomy	(X)	(X)	(X)	(X)
ALTWEB	6/27/2016	2006-2016	First surgery, Second Surgery, Atrial fibrillation induction, nanoparticle injection	atrial fibrillation, arrhythmia, nanoparticle injection survival second thoracotomy	(X)	(X)	(X)	(X)

2. Replacement. Describe the replacements that have been incorporated into this work, the replacements that have been considered but cannot be used, and the reason(s) that further replacements are not acceptable.
   ► Canines have very similar physiology to humans. They are the most commonly used model for experimental AF and therefore most suitable model for this project. Smaller animals like rodents and rabbits are not suitable models for AF as their atrial chambers are too small to sustain AF, and there are significant differences in cardiac physiology between small animal species and humans. Other large animals such as pigs are not as suitable for chronic instrumentation from a behavioral standpoint. It would be difficult to record cardiac and autonomic nerves long term simultaneously in a small animal as their nerves are too small to be recorded with the materials and technique that is being utilized by the P.I. Canine autonomic nerve physiology is also very similar to humans. The P.I. also has extensive experience with long-term autonomic nerve recording in canines. We will follow AWA recommendations to minimize distress and pain.

3. Reduction. Describe how the number of animals to be used has been minimized in this protocol and explain why further reduction would disproportionately compromise the value of the data.

► Multiple interventions will be performed in the same animal in sequential fashion to reduce the number of animals used. Each animal will act as his own control (baseline recording before atrial pacing or denervation by injection of nanoformulated botox or calcium chloride). Therefore, the need for a separate control group is eliminated.

4. Refinement. Describe the refinements that have been incorporated into this work and explain why no further refinements are feasible.
   ► The following refinements to the protocol have been made:
   (a) Performing first and second left sided thoracotomies on different intercostal spaces to aid wound healing minimize reinjury.
   (b) Avoiding or minimizing the extent of pericardiotomy (incising the pericardium) during first surgery will prevent pericardial inflammation and adhesions, and aid recovery from surgery, and reducing the need to release adhesions during second surgery, overall making surgery less invasive.
   (c) Avoiding any implantable devices other than DSI will minimize the chance of wound or pocket infections.
   (d) During injection of nanoparticles into GPs which are located in epicardial fat pads near the pulmonary vein-atrial junction, making sure a beveled needle is used with a bend to inject into epicardial fat and avoid directly puncturing the atrium.
   (e) Minimizing the release of adhesions during second surgery to avoid irreversible lung, cardiac and vascular injuries.

Describe how it was determined that the proposed work does not unnecessarily duplicate work already documented in the literature.

► The proposal serves as (a) an experimental validation of nanoparticle formulation as a slow release method of administering a pharmaceutical agent, (b) an experimental validation of the use of calcium chloride as a neurosuppressant, and (c) a form of backwards mechanistic validation of pilot clinical studies that have used botox alone (no nanoformulation) in patients with postoperative AF. All of these studies are thus novel.

X. **Other Regulatory Considerations**.

1. **Controlled drugs**.

a. Complete the table below for each drug that is used in animals on this protocol and that is classified as a controlled substance by the DEA. See ACORP Instructions, for explanations about the information requested.

Controlled substances	Storage		Personnel Authorized to Access	Location for Use		Procurement	
	Double-locked	Not Double-locked*		VA Property	Not on VA Property	VA Phar-macy	Non-VA
Buprenorphine	(X)	( )*	[redacted]	(X)	( )	(X)	( )
Pentobarbital	(X)	( )*	[redacted]	(X)	( )	(X)	( )
Brevital	(X)	( )*	[redacted]	(X)	( )	(X)	( )

			[redacted]				
Diazepam	(X)	( )*	[redacted]	(X)	( )	(X)	( )

*For any controlled substance that will NOT be stored under double lock, with limited access, describe how it will be stored, and explain why this is necessary.
►

b. Check each statement below that applies, to confirm that all controlled substances used on this protocol will be procured according to VA pharmacy policies:

► ( ) Some controlled substances will used on VA property, and all of these will be obtained through the local VA pharmacy.

► ( ) Some controlled substances will not be obtained through the local VA pharmacy, but none of these will be used on VA property. See the ACORP Instructions, for further information.

► ( ) Other. Explain►

2. **Human patient care equipment or procedural areas**. Does this protocol involve use of any human patient care equipment or procedural areas?

► ( ) Yes, some human patient care equipment or procedural area(s) will be used for the animal studies on this protocol. Check "Appendix 7" in Item Y, below, and complete and attach Appendix 7, "Use of Patient Procedural Areas for Animal Studies".

► (X)No human patient care equipment or procedural areas will be used for the animal studies on this protocol.

3. **Explosive agents**. Does this protocol involve use of any explosive agent?

► ( ) Yes, some explosive agent(s) will be used on this protocol. Check "Appendix 3" and "Appendix 8" in Item Y, below, and complete and attach Appendix 8, "Use of Explosive Agent(s) within the Animal Facility or in Animals", as well as Appendix 3, "Biosafety".

► (X)No explosive agent(s) will be used as part of this protocol.

Y. **Summary of Attachments.** To assist the reviewers, summarize here which of the following apply to this ACORP.

Appendices. Indicate which of the Appendices are required and have been completed and attached to this protocol. Do not check off or attach any appendices that are not applicable to this ACORP.

► ( ) Appendix 1, "Additional Local Information"
► ( ) Appendix 2, "Antibody Production"
► (X) Appendix 3, "Biosafety"
► (X) Appendix 4, "Ante-mortem Specimen Collection"
► (X) Appendix 5, "Surgery"
► (X) Appendix 6, "Special Husbandry and Procedures"
► ( ) Appendix 7, "Use of Patient Care Equipment or Areas for Animal Studies"

►( ) Appendix 8, "Use of Explosive Agent(s) within the VMU or in Animals"
►( ) Appendix 9, "Departures from "Must" and "Should" Standards in the *Guide*"

Standard Operating Procedures (SOPs). List in the table below, each of the SOPs referred to in this protocol, providing the information requested for each one. The approved SOPs must be included when the approved ACORP and Appendices are submitted for Just-in-Time processing before release of VA funding support.

Item	SOP		Approval Date
	Title	ID	
C.2.c			
M.1			
M.2			
U.4.a			
U.4.b			
V			

Z. **Certifications.** Signatures are required here for any ACORP that is to be submitted to VA Central Office in support of an application for VA funding. Include the typed names and dated signatures as shown below for the Main Body of the ACORP and for each of the Appendices that apply to this protocol. Do NOT include signatures for, or attach, any appendices that do NOT apply.

1. **Main Body of the ACORP.**

a. **Certification by Principal Investigator(s):**

I certify that, to the best of my knowledge, the information provided in this ACORP is complete and accurate, and the work will be performed as described here and approved by the IACUC. I understand that IACUC approval must be renewed at least annually, and that the IACUC must perform a complete *de novo* review of the protocol at least every three years, if work is to continue without interruption. I understand further that I am responsible for providing the information required by the IACUC for these annual and triennial reviews, allowing sufficient time for the IACUC to perform the reviews before the renewal dates, and that I may be required to complete a newer version of the ACORP that requests additional information, at the time of each triennial review.

I understand that further IACUC approval must be secured before any of the following may be implemented:

- Use of additional animal species, numbers of animals, or numbers of procedures performed on individual animals;

- Changing any procedure in any way that has the potential to increase the pain/distress category to which the animals should be assigned, or that might otherwise be considered a significant change from the approved protocol;
- Performing any additional procedures not already described in this ACORP;
- Use of any of these animals on other protocols, or by other investigators.

I further certify that:

- No personnel will perform any animal procedures on this protocol until the IACUC has confirmed that they are adequately trained and qualified, enrolled in an acceptable Occupational Health and Safety Program, and meet all other criteria required by the IACUC. When new or additional personnel are to work with the animals on this protocol, I will provide this information to the IACUC for confirmation before they begin work;
- I will provide my after-hours contact information to the animal care staff for use in case of emergency.

Name(s) of Principal Investigator(s)	Signature	Date
[redacted]	[redacted]	07/18/2017

b. **Certification by IACUC Officials.**

We certify that:

- We, with the IACUC, have evaluated the care and use of animals described on this ACORP, in accordance with the provisions of the USDA Animal Welfare Act Regulations and Standards, PHS Policy, the *Guide for the Care and Use of Laboratory Animals*, and VA Policy;
- The IACUC has determined that the care and use of animals described in this ACORP is appropriate, and has therefore approved the protocol;
- The full text of any minority opinions is documented here as indicated below:

► ( ) No minority opinions were submitted by any IACUC participant for inclusion.

► ( ) Minority opinions submitted by IACUC participants are copied here
►

► ( ) Minority opinions submitted by IACUC participants are attached on separate pages labeled “IACUC Minority Opinion” (indicate the number of pages►)

Name of Attending Veterinarian (VMO or VMC)	Signature	Date

Name of IACUC Chair	Signature	Date

2. **Appendix 2. Antibody Production.** No signatures required.

3. **Appendix 3. Biosafety.**

   a. **Certification by PI(s) and IACUC Officials:**

   We certify that:

   - Before any animal experiments involving hazardous agents (identified in Item 10.a of Appendix 3) are performed, SOPs designed to protect all research and animal facility staff as well as non-study animals will be developed and approved by the appropriate VA or affiliated university safety committee and by the IACUC;

   - All personnel who might be exposed to the hazardous agents (identified in Item 10.a of Appendix 3) will be informed of possible risks and will be properly trained ahead of time to follow the SOPs to minimize the risks of exposure.

Name(s) of Principal Investigator(s)	Signature(s)	Date
[redacted]	[redacted]	[redacted]
Name of Institutional Veterinarian	Signature	Date
Name of IACUC Chair	Signature	Date

   b. **Certification by Biosafety Official.** I certify that:

   - Each agent to be administered to animals on this protocol has been properly identified in Item 1 of Appendix 3 as to whether it is “toxic”, “infectious”, “biological”, or “contains recombinant nucleic acid”;

   - The use of each of the agents thus identified as “toxic”, “infectious”, or “biological”, or “contains recombinant nucleic acid” is further documented as required in Items 4, 5, 6, and/or 8, as

applicable, and in Item 10.a of Appendix 3;

- ☐ The use of each of these agents has been approved by the appropriate committee(s) or official(s), as shown in Item 10.a of Appendix 3.

Name of the Biosafety Officer, or of the Chair of the Research Safety or Biosafety Committee	Signature	Date

c. **Certification by Radiation Safety Official.** I certify that:

- ☐ Each agent to be administered to animals on this protocol has been properly identified in Item 1 of Appendix 3 as to whether it is "radioactive";
- ☐ The use of each radioactive agent is further documented as required in Items 7 and 10.a of Appendix 3;
- ☐ The use of each radioactive agent has been approved by the appropriate committee(s), as shown in Item 10.a of Appendix 3.

Name of the Radiation Safety Officer, or of the Chair of the Radiation Safety or Isotope Committee	Signature	Date

4. **Appendix 4. Ante-mortem Specimen Collection.** No signatures required.

5. **Appendix 5. Surgery. Certification by the PI(s).** I certify that:

- ☐ To the best of my knowledge, the information provided in Appendix 5 of this ACORP is complete and accurate;
- ☐ The surgical procedures will be performed and the post-operative care (including administration of post-operative analgesics) will be provided as described;
- ☐ The spaces where any survival surgical procedures will be performed (listed in Item 4 of Appendix

5) are suitable for sterile/aseptic surgery;

- The names and contact information for research personnel to notify or consult in case of emergencies will be provided to the VMU supervisor and veterinary staff;
- Post-operative medical records will be maintained and readily available for the veterinary staff and the IACUC to refer to, and will include the following:

  - Identification of each animal such that care for individual animals can be documented.
  - Daily postoperative medical records for each animal, that include documentation of daily evaluation of overall health and descriptions of any complications noted, treatments provided, and removal of devices such as sutures, staples, or wound clips;
  - Documentation of the administration of all medications and treatments given to the animals, including those given to reduce pain or stress.
  - Daily records covering at least the period defined as "post-operative" by local policy.
  - The signature or initials of the person making each entry.

.

Name(s) of Principal Investigator(s)	Signature(s)	Date
[redacted]	[redacted]	[redacted]

6. **Appendix 6. Special Husbandry and Procedures.** No signatures required.

7. **Appendix 7. Use of Patient Care Equipment or Areas for Animal Studies.**

   a. **Certification by the Principal Investigator(s).** I certify that, to the best of my knowledge, the information provided in Appendix 7 of this ACORP is complete and accurate, and the use of patient care equipment or areas for these animal studies will be as described.

Name(s) of Principal Investigator(s)	Signature(s)	Date

   b. **Certification by the officials responsible for the use of any human patient care equipment in animal procedural areas.** Each of the following must sign to indicate that they have granted approval for the human patient care equipment to be moved to the VMU or other animal procedural

area to be used on animals and then returned to the human patient care area, as described in Appendix 7. Leave this section blank, if not applicable.

Name of IACUC Chair	Signature	Date
Name of the Manager of the Human Patient Care Equipment	Signature	Date

c. **Certification by the officials responsible for the use of the equipment in human patient care areas for these animal studies.** Each of the following must sign to indicate that they have granted approval for animals to be transported into human patient care areas for study or treatment, as described in Appendix 7. Leave this section blank, if not applicable.

Name of IACUC Chair	Signature	Date
Name of Attending Veterinarian (VMO or VMC)	Signature	Date
Name of the Chair of the Clinical Executive Board, or the Service Chief responsible for the Patient Care Area and Equipment	Signature	Date
Name of ACOS for R&D	Signature	Date
Name of Chief of Staff	Signature	Date
Name of Director or CEO of the Facility (Hospital or Clinic)	Signature	Date

8. **Appendix 8. Use of Explosive Agent(s) within the Animal Facility or in Animals.**

a. **Certification by the Principal Investigator(s).**

I certify that, to the best of my knowledge, the information provided in Appendix 8 of this Animal Component of Research Protocol (ACORP) is complete and accurate, and the use of explosive agents in these animal studies will be as described.

I further certify that:

- Procedures involving explosive agent(s) will be performed within a properly operating, ventilated safety hood;
- All electrical equipment operating when explosive agent(s) are in use will be positioned and powered outside of the hood;
- Once the seal is broken on any containers of explosive agents, they will be kept in a safety hood throughout use, stored in an explosion-proof refrigerator or other approved storage area, and discarded properly once completely emptied;
- Proper procedures will be used for safe and appropriate disposal of items (including animal carcasses) that may contain residual traces of the explosive agent(s).

Name(s) of Principal Investigator(s)	Signature(s)	Date

b. **Certification by the officials responsible for overseeing the use of explosive agent(s) in this protocol.** Each of the following must sign to verify that they or the committee they represent have granted approval.

Name of IACUC Chair	Signature	Date
Name of Attending Veterinarian (VMO or VMC)	Signature	Date
Name of Safety/Biosafety Officer for the Facility	Signature	Date

Name of ACOS for R&D	Signature	Date
Name of VISN Regional Safety Officer	Signature	Date

1. **Departures from "Must" and "Should" Standards in the *Guide*.** No signatures required.

ACORP APPENDIX 3
BIOSAFETY
VERSION 4

See ACORP App. 3 Instructions, for more detailed explanations of the information requested.

1. **Summary of <u>All</u> Materials Administered to Animals on this Protocol.** Complete the table below for <u>all</u> materials to be administered to any animal on this protocol, indicating the nature of the material by marking EVERY box that applies, and indicating the BSL number for any infectious agents:

Material (Identify the specific agent, device, strain, construct, isotope, etc.)	**Source** (Identify the vendor or colleague, or specify which animals on this protocol will serve as donors)	**Nature of Material**						
		Toxic Agent (Item 4)	nfectious Agent (Item 5) -- Enter the CDC Biosafety Level (BSL 1, 2, 3, or 4)	Biological Agent (Item 6)	Radioactive Agent (Item 7)	Contains Recombinant Nucleic Acid (Item 8)	Routine Pre- or Post-Procedural Drug	Euthanasia agent
Pentobarbital	VA Pharmacy	( )	( )BSL_	( )	( )	( )	(X)	( )
Metoclopromide	VA Pharmacy							
Data Sciences International radiotelemety device	Data Sciences International	( )	( )BSL_	( )	( )	( )	(X)	( )
Isoflurane	Richmond VA pharmacy	( )	( )BSL_	( )	( )	( )	(X)	( )
Brevital	Richmond VA pharmacy	( )	( )BSL_	( )	( )	( )	(X)	( )
Buprenorphine	Richmond VA pharmacy	( )	( )BSL_	( )	( )	( )	(X)	( )
Diazepam	Richmond VA pharmacy	( )	( )BSL_	( )	( )	( )	(X)	( )
Acepromazine	Butler Schein	( )	( )BSL_	( )	( )	( )	(X)	( )
Carprofen	Butler Schein	( )	( )BSL_	( )	( )	( )	(X)	( )
Baytril	Bayer	( )	( )BSL_	( )	( )	( )	(X)	( )
Penicillin	Butler Schein							
Bismuth Subsalicylate	Local pharmacy	( )	( )BSL_	( )	( )	( )	(X)	( )
Famotidine	Local pharmacy	( )	( )BSL_	( )	( )	( )	(X)	( )

Meloxicam	Butler Schien	( )	( )BSL_	( )	( )	( )	(X)	( )
Epinephrine	Richmond VA pharmacy	( )	( )BSL_	( )	( )	( )	(X)	( )
Amiodarone	Richmond VA pharmacy	( )	( )BSL_	( )	( )	( )	(X)	( )
Cefpodoxime	Richmond VA pharmacy	( )	( )BSL_	( )	( )	( )	(X)	( )
Calcium Chloride	NanoTech			()				
Nanoparticle Botox	NanoTech			(X)				
Nanoparticle Calcium Chloride	NanoTech							
Vetericyn	Butler Schien							
Clonidine	Richmond VA pharmacy	(X)	( )BSL_	( )	( )	( )	( )	( )
Phenylephrine	Richmond VA pharmacy	(X)	( )BSL_	( )	( )	( )	( )	( )

2. **Summary of How Materials will be Administered.** Complete the table below for each of the materials shown in the table in Item 1 above:

Material* (Identify the specific agent, device, strain, construct, isotope, etc.)	**Dose** (e.g., mg/kg, CFU, PFU, number of cells, mCi) and **Volume** (ml)	**Diluent* or Vehicle***	**Route** of admin	**Frequency or duration** of admin	**Reason** for Administration and **Expected Effects**	Location of **Further Details** in this ACORP (specify "Main Body" or "App #", and identify the Item)	Administration Under **Anesthesia, sedation, or tranquilization** (Y/N)
Pentobarbital	30mg/kg total (to effect) or 100mg/kg for euthanasia	None	IV	Once per surgery, if brevital not available Or once for euthanasia	Anesthetic	Pg 5,20,38, Apendix 5, 1st Surgery, Final surgery, euthanasia	Y
Data Sciences International radiotelemety device	1 Device	None	SC implantation during first surgery	Once	To monitor vitals	Pg 3,4,5 1st Surgery	Y
Baytril	6-10mgkg	None	Oral	7-10 days post surgery	Antibiotic	Pg 5, 1st&2nd Surgery Appendix 5	N
Isoflurane	1-4%	None	Inhalation	Duration of surgery	Anesthesia	Pg 5, 1st&2nd Surgery Appendix 5	Y
Brevital	6-10 mg/kg	Saline	IV	Once	Anesthesia	Pg 5, 1st&2nd Surgery Appendix 5	Y
Buprenorphine	0.01-0.02 mg/kg	None	IM	Q8-12hr	Analgesics	Pg 5, 1st&2nd Surgery Appendix 5	N

Diazepam	0.2-2.0 mg/kg	None	Oral or IM	Once for 1 day	Calm during post-op to keep sutures intact and reduce risk of infeciton	Pg 5, 1st&2nd Surgery Appendix 5	N
Acepromazine	0.05-0.1mg/kg	None	IV and Oral	Once	sedative	Pg,5, 6, 36, 1st&2nd Surgery Appendix 5	N
Carprofen	2 mg/kg	None	Oral	SID up to three days	Anti-inflammatory and pain relief during post-op	Pg 5, 1st&2nd Surgery Appendix 5	N
Baytril	6-10mgKg	None	Oral	SID for 10 Days post surgery	Antibiotics	Pg 5, 1st&2nd Surgery Appendix 5	N
Penicillin	900,000 units	None	IM	Once	Antibiotic	Pg 5, 1st Surgery Appendix 5	N
Famotidine	0.5-1.0mg/kg	None	Oral or IV	Once a day as needed	Appetite recovery	Pg 5, 1st&2nd Surgery Appendix 5	N
Meloxicam	0.2 mg/kg	none	IM or SQ	Once or as an alternative for Carprofen	Pain Relief	Pg 5, 1st&2nd Surgery Appendix 5	N
Epinephrine	Low dose (0.01 mg/kg) high dose (0.1 mg/kg)	Normal Saline	IV	every 3–5 min early in resuscitation efforts; after prolonged resuscitation efforts (15 mins)	Resuscitation efforts	Pg 5, 1st Surgery Appendix 5	Y
Amiodarone	7mg/kg	None	IV	Single dose, repeated every 5 minutes if necessary	Resuscitation	Pg 5, 1st&2nd Surgery Appendix 5	Y
Cefpodoxime	5 mg/kg	None	Oral	SID 10 days post surgery	Antibiotic	Pg 5, 1st&2nd Surgery Appendix 5	N

Nanoparticle Botox	50 U/ml of botox per GP and not exceed 200U per dog. 1ml injected per GP	Nanopure water	GP injection	Once during Surgery	Suppression of AF induction	Pg 5, 1st&2nd Surgery Appendix 5	Y
Nanoparticle Calcium Chloride	5mM of CaCL2. 1 ml injected per GP	Nanopure water	GP injection	Once during Surgery	Suppression of AF induction	Pg 5, 1st&2nd Surgery Appendix 5	Y
Calcium Chloride	40 micromolar solution in 1 to 4 mls	Nanopure water	GP injection	Once during surgery	Suppression of AF induction	Pg 5, 1st&2nd Surgery Appendix 5	Y
Metoclopromide	0.2-0.5 mg/kg		IV	PRN	antiemetic		N
Vetericyn	2-3 sprays	None	Topical	Once after surgery	To promote wound healing	Main body	N
Clonidine	10 µg/kg	Dextrose 5%	IV	Twice in 2 months (1st at week 2 post-op & 2nd after week 1 of 2nd surgery post op)	Assess correlation between renal and cardiac sympathetic nerves. Transient (minutes) drop in BP, HR	Pg 9	N
Phenylephrine	0.01mg/kg	Dextrose 5%	IV	Twice in 2 months (1st at week 2 post-op & 2nd after week 1 of 2nd surgery post op)	Assess correlation between renal and cardiac sympathetic nerves. Transient (minutes) drop in BP, HR	Pg 9	N

*Each material, diluent, or vehicle that is listed as FDA approved or is labeled "USP" is pharmaceutical grade. Check on-line for formulations that are FDA approved for administration to humans (http://www.fda.gov/Drugs/InformationOnDrugs/ucm129662.htm) or animals (http://www.fda.gov/AnimalVeterinary/Products/ApprovedAnimalDrugProducts/UCM042847). Designate with a * each material and each diluent or vehicle to be used that is not pharmaceutical grade. For each of these, explain here why the use of a non-pharmaceutical grade formulation is necessary, and describe how it will be ensured that the material is suitable for use. (See ACORP App. 3 Instructions, for specifics about the level of detail required.)

3. **Anesthesia, Sedation, or Tranquilization.** Complete 3.a. and 3.b. below:

a. For each material with "Y" entered in the last column of the table in Item 2 above, describe the anesthesia, sedation, or tranquilization to be used, identifying the anesthetic, sedative, or chemical tranquilizer, and

detailing the dose, volume, and route of administration (Make sure that these agents are also included in Item 1 of this appendix, as materials to be administered):
►
Acepromazine (0.5-2.0 mg/kg PO) is given prior to the Brevital (or Pentobarbital) administration via IV catheter. The animal is sedated with Brevital or Pentobarbital prior to isoflurane administration. The DSI is implanted under isoflurane anesthesia. The Botox and Nanoparticles are injected under isoflurane anesthesia.

b. For each material with "N" entered in the last column of the table in Item 2 above, explain why no anesthesia, sedation, or tranquilization is necessary, or can be provided, and describe any alternate methods of restraint that will be used.
►
All agents given without anesthesia are administered via IM, IV or oral routes. Injections require no anesthesia as only momentary pain is experienced and none of the agents are irritating to the tissues. Agents given orally can be hidden in treats provided by the VMU or provided in a flavor tab that is eaten voluntarily by the canine.

4. **Toxic Agents.** Complete the table below for each of the materials listed as a "toxic agent" in the table in Item 1 above, checking the all of the properties that apply (see ACORP App. 3 Instructions, for details).

Name of Toxic Agent	a. Mutagen	b. Carcinogen	c. Teratogen	d. Select Agent?			e. Other – specify toxic properties
				Not a Select Agent	Select Agent Used in Sub-threshold Quantities	Select Agent that Requires Registration/Approval	
Botox	( )	( )	( )	(X)	( )	( )*	(X)►Neurotoxin
Clonidine	( )	( )	( )	(X)	( )	( )*	(X)►anti-hypertensive
Phenylephrine	( )	( )	( )	(X)	( )	( )*	(X)►hypertensive

*For each "select agent" that requires registration/approval (copy the lines below for each agent):

Name of agent ►

Registered with CDC or USDA ►
Registration Number ►
Registration Date ►
Expiration Date of Registration ►

Name of official who granted approval on behalf of VACO►
Date of approval►

5. **Infectious Agents.** Complete the table below for each of the materials listed as an "infectious agent" in the table in Item 1 above (see ACORP App. 3 Instructions, for details).

Name and BSL Number of Infectious Agent	a. ABSL Number *	b. Drug Sensitivity Panel Available? (Describe)	c. Select Agent?		
			Not a Select Agent	Select Agent used in Sub-threshold quantities	Select Agent that Requires Registration/Approval
		(Yes/No)	( )	( )	( )**
		(Yes/No)	( )	( )	( )**
		(Yes/No)	( )	( )	( )**
		(Yes/No)	( )	( )	( )**
		(Yes/No)	( )	( )	( )**
		(Yes/No)	( )	( )	( )**

*Complete the following for each agent for which the ABSL Number given is less than the BSL Number shown (copy the lines below for each agent):

Name of agent ►
Justification for applying ABSL measures that are less protective than those recommended ►

**For each "select agent" that requires registration/approval (copy the lines below for each agent):

Name of agent ►

Registered with CDC or USDA ►
Registration Number ►
Registration Date ►
Expiration Date of Registration ►

Name of official who granted approval on behalf of VACO►
Date of approval►

6. **Biological Agents.** Complete the table below for each of the materials listed as a "biological agent" in the table in Item 1 above (see ACORP App. 3 Instructions, for details).

Name of Biological Agent	Screening for Infectious Agents
Botox	There is no risk of transmission of infections, viruses or cancer from Botox

7. **Radioactive Agents.** Complete the table below for each of the agents listed as a "radioactive agent" in the table in Item 1 above (see ACORP App. 3 Instructions, for details).

Name of Radioactive Agent (specify the isotope)	Authorized Individual	Approving Committee or Official

8. **Agents Containing Recombinant Nucleic Acid.** For each of the materials checked in the table in Item 1, above, as "contains recombinant nucleic acid", indicate which of the conditions applies (see ACORP App. 3 Instructions, for details).

Name of Agent that Contains Recombinant Nucleic Acid	Subject to the *NIH Guidelines for Research Involving Recombinant DNA Molecules*	Exempt
	( )	( )
	( )	( )
	( )	( )

9. **Potential for Pain or Distress**. Complete the table below for each of the agents listed in Item 1, above, that is expected to have potentially painful or distressing effects on the animals (see ACORP App. 3 Instructions, for details).

Name of Agent	Nature of Potential Pain/Distress	Measures to Alleviate Pain/Distress
Clonidine	**Transient (minutes) drop in BP, HR**	**Acepromazine is given and the drugs are very short lived**
Phenylephrine	**Transient (minutes) increase in BP**	**Acepromazine is given and the drugs are very short lived**

10. **Protection of Animal Facility Staff from Hazardous Materials.** Complete Items 10.a and 10.b, below, for each of the agents listed in the table in Item 1, above, as "toxic", "infectious", "biological", "radioactive", or "contains recombinant nucleic acid" (detailed in Items 4 – 8). This item specifically addresses members of the animal facility staff; protection of the research staff from each of these agents must be addressed in Item G of the main body of the ACORP. See ACORP App.3 Instructions, for details.

a. Complete the table below.

Name of Hazardous Agent	Approving Committee or Official	Institution (VA or affiliate)	Names of Animal Facility Staff Members at Risk

Botox	**SRS**	**VA**	**No staff members are at risk**
Clonidine	**SRS**	**VA**	**No Staff member is at risk**
Phenylephrine	**SRS**	**VA**	**No Staff member is at risk**

b. Detail how the individuals listed in the table above (Item 10.a.) have been (or will be) informed of the possible risks of exposure, and have been (or will be) trained to avoid exposure to these agents.
►There is no risk to staff members.

11. **Signatures.** Provide the applicable signatures on the signature pages (Item Z.3) of the main body of this ACORP.

ACORP Appendix 4
ANTEMORTEM SPECIMEN COLLECTION
VERSION 4

See ACORP App. 4 Instructions, for more detailed explanations of the information requested.

1. **Summary.** Complete the table below for each specimen to be collected from a live animal on this protocol (see ACORP App. 4 Instructions, for details).

Specimen Collected	Site and Method of Collection	Anesthesia (Yes/No)	Amount Collected Each Time	Volume Replacement (Yes/No/NA)	Total Number of Collections per Animal	Time Intervals Between Successive Collections
Blood	**Brachial or Jugular vein/ Phlebotomy**	**No**	**10-15 ml (< 1%)**	**No**	**4**	**3-4 weeks**
Blood	**During first thoracotomy via aorta**	**Yes**	**<10ml**	**Y**	**1**	**once**

2. **Use of Anesthetics, Tranquilizers, or Analgesics**.

a. For each specimen described in Item 1, above, as being collected WITHOUT anesthesia, complete Items 2.a(1) and 2.a(2), below:

(1) Explain why no measures will be taken to prevent pain (e.g., because of scientific requirements described here, or because the collection method involves no more than minor or momentary pain).
► Blood draw is a relatively painless procedure. The animal may experience brief and minimal discomfort from catheter insertion, however not enough that would require any pain relief medication.

(2) Completely describe any method of physical restraint that may be used.
► Blood draws will always be conducted with 2 research personnel present; one person to manually restrain the animal and the other person to draw the blood. This ensures this procedure is carried out safely for both personnel and the animal.

b. For each specimen described in Item 1, above, as being collected WITH anesthesia, complete the following table:

Anesthetic, tranquilizer, or analgesic agent	Dose (mg/kg) and volume (ml)	Route of administration	Frequency of administration
Isoflurane inhalation	**1-3%**	**Vaporizer**	**continuously**

3. **Volume Replacement for Fluid Collections.**

a. For each fluid specimen described in Item 1, above, for which NO volume replacement will be provided, explain why not.
► The blood draw from the veins is not a significant volume to warrant replacement. During the surgery, they are on continuous IV fluids to replace any volume lost.

b. For each fluid specimen described in Item 1, above, for which volume replacement WILL be provided, describe the replacement fluids that will be administered (including their composition, volume, and route of administration).
►

4. **Monitoring the animals.** Detail how the animals will be monitored after collection of specimens to ensure that they recover appropriately (see ACORP App. 4 Instructions, for details).
► Pressure will be applied to site of blood draw until bleeding has ceased.

ACORP Appendix 5
SURGERY
VERSION 4

See ACORP App. 5 Instructions, for more detailed explanations of the information requested.

1. **Surgery Classification.** Complete the table below for each surgery included in this protocol, and indicate how it is classified (terminal, minor survival, major survival, one of multiple survival). See ACORP App. 5 Instructions, for details.

Surgery		Terminal	Survival		
#	Description (specify the species, if ACORP covers more than one)		Minor	Major	One of Multiple*
1	First surgery & Data Sciences International (DSI) nerve recording device implantation	( )	( )	(X)	(X)*
2	Second Surgery (Nanoparticle Injection)	( )	( )	(X)	(X)*
3	Final Surgery	(X)	( )	( )	(X)*
4	Wound Revision	( )	(x)	( )	(X)*

*If survival surgery (including major surgeries and any minor surgeries that may induce substantial post-procedural pain or impairment) will be performed as part of this protocol in addition to any other such surgery (on this or another protocol) on the same individual animal, complete items 1.a and 1.b, below:

a. Provide a complete scientific justification for performing the multiple survival surgeries on an individual animal:
► The importance of multiple surgeries is because of the need for each animal to serve as its own internal controls. Nerve activity varies between animals; therefore comparison between animals is technically limited. Thus, each animal will need to have baseline measurements before injection of botox/calcium chloride for denervation, and a second surgery for denervation procedures.

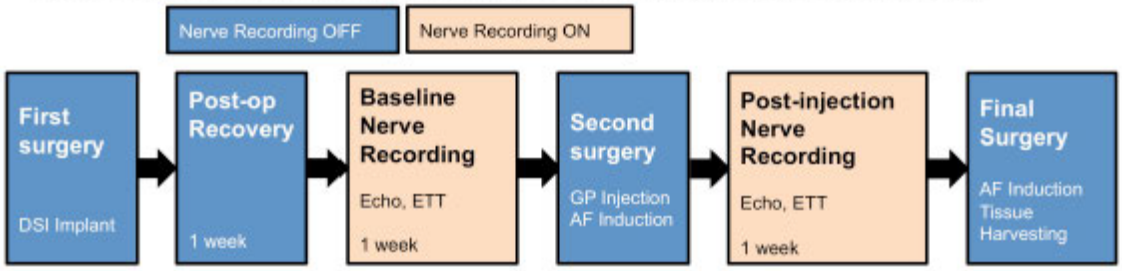
Alternatively, using a single survival surgery necessitates having a control group, and comparing between control and experimental animals.

To aid in healing, first surgery will involve a limited thoracotomy in the 3rd intercostal space simply for implantation of DSI device. Second surgery will be a limited thoracotomy in a different interspace. Wound revision- it is not uncommon for surgical wound dehiscence to occur immediately after surgery. A minor and quick procedure would be needed to correct any defect**.**

b. Give the interval(s) between successive surgeries, and the rationale for choosing the interval(s):
► Animals will be given 21 days to recover between the first and second surgery. This is done so that the animal has time to fully recover, and baseline recordings can be completed before the second survival surgery.
Wound revision- This would occur 2-7 days after the initial surgery if needed.

2. **Description of Surgeries.** Describe each surgery listed in Item 1, providing enough detail to make it clear what the effects on the animal will be. (Pre-operative preparation, anesthesia, and post-operative recovery will be covered in items 5, 6, and 7, below.)

Figure 1. Experimental Protocol. Events occur in sequence from left to right.



Surgery 1 ►
The canine is pre-anesthetized with Acepromazine 0.05-0.1mg/kg approximately 1 hour before surgery. They are given Buprenorphine 0.01-0.02 mg/kg IM, Penicillin (900,000 units) IM and Famotidine 0.5-1 mg/kg PO prior to being anesthetized. They are anesthetized with Brevital 6-10 mg/kg IV (Pentobarbital 30mg/kg IV can be given if Brevital is unavailable) to effect to allow for intubation with a cuffed endotracheal tube. The endotracheal tube is then connected to a vaporizer and respirator for isoflurane induction and mechanical ventilation. Isoflurane 1-3 % mixed with oxygen is used for surgical plane of anesthesia throughout the surgery unless otherwise described. Heart rate, blood pressure and temperature will be recorded every 15 minutes. Average heart rate for the dogs under anesthesia is 85-100 bpm. If heart rate increases by more than 5bpm from the average to this point, isoflurane may be temporarily increase by .5% (no more than 3% during surgery). Each dog will be weighed before surgery to determine the maximum dose of anesthetic that is allowed to prevent them from overdose

Surgery will be performed under full aseptic technique. We will perform a left lateral thoracotomy incision at the T2-3 level. We will collect blood sample (less 10 mls) for analyses of norepinephrine levels from the aorta. We will implant a Data Sciences International (DSI) radiotelemetry device subcutaneously in canines. This device has three bipolar channels. We will implant one channel to record the left stellate ganglion nerve activity and one channel to record cardiac vagal nerve activity plus ECG. The third channel will be reserved for recording ganglionated plexi (GP) nerve activity. GP are located in epicardial fat pads. The electrode will be sutured onto the epicardial fat pad (but not onto atrial muscle underlying it). Occasionally, there is a risk of injuring the underlying atrial muscle resulting in bleeding. This bleeding will be quickly controlled and if necessary, sutured close with a non-cutting vicryl or silk suture. If this bleeding is not controllable, the animal will be euthanized by exsanguination under anesthesia.
In order to most accurately view autonomic nerve activity during an EP studies isoflurane may need to be turned off for a period of 30 minutes. We will first attempt to conduct the EP studies with isoflurane but if the nerve activity is altered due to this anesthetic, the following protocol will be followed. Pentobarbital will be administered as follows to keep the animal in a surgical plane of anesthesia. We will give 4-5mg/kg of pentobarbital initially and then turn off the isoflurane. The heart rate will be monitored closely. Average heart rate for the dogs under anesthesia is 85-100bpm. If heart rate increases by more than 5bpm from the average to this point, small doses of pentobarbital (2-3 mg/kg not to exceed a total 30 mg/kg including the loading dose). Once the nerve recording is finished, isoflurane will be resumed until end of surgery. Isoflurane has a nature of suppressing nerve activity and does so more than pentobarbital. For this reason, isoflurane is removed. If the maximum dose of pentobarbital is reached during surgery, isoflurane must be resumed and the EP study completed under the effects of pentobarbital. The DSI leads will be rechecked for stability, and the device will be implanted in a subcutaneous extrathoracic pocket. Once the device has been implanted in the pocket and the lead positions have been verified we will begin closing all surgical sites.

Some subjects will be transferred to protocols # 02289 a minimum of ten days following surgical thoracotomy for implantation of a neurophysiologic and electrophysiologic recording device. After 10

days animals will be evaluated for transfer to protocol #02289. The parameters assessed will include return of baseline function and activity; normal eating, sleeping and elimination behaviors; and minimal or no requirement for pain control medications, Additionally, the canine will need to have no weight loss for at least 3 days prior to transfer. Subjects will be returned to this protocol baring any physical impairments mentioned above, after the completion of procedures on protocol #02289.

EP study and AF induction. The purpose of the EPS is to determine the effective refractory period (ERP) of the atrium and inducibility of AF. A second purpose is to identify the GP by high frequency (20Hz) subthreshold stimulation. ERP and AF inducibility will be determined by programmed stimulation (single, double, triple premature stimuli) from three different sites in the LA (left atrial appendage, posterior and anterior LA). The refractory period is the longest coupling interval of the premature beat that fails to capture the tissue. A short refractory period is a substrate for AF. Vulnerability to induced AF is assessed by sequential single, double and triple extrastimuli down to refractory period. Refractory period and vulnerability to AF are the primary end-points to be assessed across multiple surgeries, allowing comparison of the baseline state, vs a three-week postoperative state before and after administration of nanoformulated therapy to GP. High frequency subthreshold stimulation at 20Hz, at an output that does not capture atrium, is performed at the sites of GP. A positive response is defined by a change in heart rate of 5% or more after 10 sec of stimulation.
We will be using Bloom electrical stimulator. Programmed atrial stimulation will not result in comparable high ventricular rates because of AV node protection. The high frequency stimulation of GPs is a subthreshold stimulation meant to stimulate autonomic nerves, without stimulating the heart. In our experience [redacted] we expect any AF to be induced will be non-sustained (lasting a matter of seconds to minutes only). If AF does not self terminate, we will perform burst overdrive pacing to terminate AF. In our experience, this is always successful. Thus, no animal will be left in AF at the end of the procedure. Each EPS typically lasts 30 minutes.

Average heart rate for the dogs under anesthesia is 85-120 bpm. If heart rate increases by more than 5bpm from the average to this point, isoflurane may be temporarily increase by .5% (no more than 3% during surgery). Each dog will be weighed before surgery to determine the maximum dose of anesthetic that is allowed to prevent them from overdose.
The DSI leads will be rechecked for stability, and the device will be implanted in a subcutaneous extrathoracic pocket. Once the device has been implanted in the pocket and the lead positions have been verified we will begin closing all surgical sites. We will then proceed to closing chest wound. First the thoracotomy site will be closed with surgical steel wires to hold the ribs together. Then the overlying intercostal muscles and deep muscle layers will be closed with interrupted Vicryl sutures. Once this has been closed a previously implanted chest tube will be used to evacuate all air from the pleural cavity and reinflate the left lung. The skin will then be closed in two layers using running Vicryl suture lines. Finally reinforcing nylon vertical mattress sutures will be placed to hold the wound closed during the initial healing phase.
We will close the thoracotomy using silk sutures. The overlying muscle and skin will be closed in multiple layers using interrupted and continuous sutures.
Heart rate, blood pressure, and temperature are monitored and recorded every 15-30 minutes for the duration of this procedure.
Animals are allowed to recover on the ventilator with 100% oxygen until swallowing reflex is noted. The incision is sprayed with a Vetericyn gel to promote wound healing. The endotracheal tube is removed and the canine is moved to a recovery cage with blankets and a warming pad. Once sternal, they receive meloxicam 0.2mg/kg IM.
The canine remains in the post-operative recovery cage until the following morning when they are moved to the standard chain link run with padding and blankets. They are given buprenorphine 0.01-0.02 mg/kg IM twice a day for three days for analgesia. Carprofen (2mg/kg PO) can be given for 1-3 days if pain is noted after the buprenorphine regimen is completed. Famotidine 0.5-1.0 mg/kg PO or IV

can be given as needed for nausea or appetite stimulant (metoclopramide 0.2-0.5 mg/kg IV or bismuth subsalicylate 262 mg PO, can be given if needed). Some canines are too active post operatively which prevents incision healing. In this case, they are given Diazepam 0.2-2mg/kg IM or PO twice a day as needed Cefpodoxime 5mg/kg PO is given once a day for 10 days to prevent wound infection. If there is ongoing concern for wound infection while receiving Cefpodoxime (Baytril 5-20mg/kg PO can be given once a day for 10 days). IV Fluids are available with or without 5% dextrose if an animal has not resumed normal eating habits within 2 days. . Canine weights will be observed and recorded every other day while on antibiotics and then twice a weekly thereafter.

There is a very small risk of spontaneous ventricular fibrillation during the left thoracotomy surgery. Should this happen there are sterile internal defibrillator paddlers connected to a defibrillator set at 50 Joules prepared for resuscitation efforts. Epinephrine will also be administered at a low dose (0.01 mg/kg) will be given every 3–5 min early in resuscitation efforts; a high dose (0.1 mg/kg) will be given after prolonged effort (15 minutes) with no response. Amiodarone (7mg/kg) will be used as an alternative. This is administrated in a single dose and repeated as needed every 5 minutes.

Surgery 2 ►

The canine is pre-anesthetized with Acepromazine 0.05-0.1mg/kg approximately 1 hour before surgery. They are given Buprenorphine 0.01-0.02 mg/kg IM, Penicillin (900,000 units) IM and Famotidine 0.5-1 mg/kg PO prior to being anesthetized. They are anesthetized with Brevital 6-10 mg/kg IV (Pentobarbital 30mg/kg IV can be given if Brevital is unavailable) to effect to allow for intubation with a cuffed endotracheal tube. The endotracheal tube is then connected to a vaporizer and respirator for isoflurane induction and mechanical ventilation. Isoflurane 1-3 % mixed with oxygen is used for surgical plane of anesthesia throughout the surgery unless otherwise described. Heart rate, blood pressure and temperature will be recorded every 15 minutes. Average heart rate for the dogs under anesthesia is 85-100 bpm. If heart rate increases by more than 5bpm from the average to this point, isoflurane may be temporarily increase by .5% (no more than 3% during surgery). Each dog will be weighed before surgery to determine the maximum dose of anesthetic that is allowed to prevent them from overdose

This will occur 21 days after first surgery. This will be followed by unilateral thoracotomy in some groups and bilateral thoracotomy in other groups to access either 2 GPS or all 4 GPs for injection of neurotoxin.

We will start with a left thoracotomy. This will be one to two interspaces lower than the one performed during first surgery. The original incision in the 3rd intercostal space will not be reaccessed. We expect to be able to limit the incision size of the present incision to about 4 cm, which will speed healing. This limited incision will allow us to directly visualize the GPs on the left side, inject them with nanoparticles, and subsequently induce AF by programmed electrical stimulation. We will take care to release adhesions to avoid lung or vascular injury. The degree of adhesions varies with the individual. If the adhesions are dense, we may avoid them and make do with a reduced surgical field, because of the risk of bleeding and lung injury. If we create lung injury, which is manifest by air leak, or bleeding, we will perform a suture ligation of the injured lung segment with vicryl or silk suture. We may need to switch from isoflurane maintenance to Pentobarbital) which will allow autonomic nerve activity to be recorded more clearly. We will attempt to record nerve activity under isoflurane anesthesia but should we need to switch to Pentobarbital we will give 4- 5mg/kg of pentobarbital at this time prior to suspension of isoflurane and the heart rate will be monitored closely. Average heart rate for the dogs under anesthesia is 85-100bpm. If heart rate increases by more than 5bpm from the average to this point, small doses of pentobarbital (2mg/kg) will be administered to effect. Each dog will be weighed before surgery to determine the maximum dose of Pentobarbital that is allowed to prevent them from overdose. Once the nerve recording is finished, isoflurane will be resumed until end of surgery. Isoflurane has a nature of suppressing nerve activity and does so more than pentobarbital. For this

reason, isoflurane is removed. If the maximum dose of pentobarbital is reached during surgery, isoflurane must be resumed and the EP study completed under the effects of pentobarbital.

EP study and AF induction. The purpose of the EPS is to determine the effective refractory period (ERP) of the atrium and inducibility of AF. A second purpose is to identify the GP by high frequency (20Hz) subthreshold stimulation. ERP and AF inducibility will be determined by programmed stimulation (single, double, triple premature stimuli) from three different sites in the LA (left atrial appendage, posterior and anterior LA). The refractory period is the longest coupling interval of the premature beat that fails to capture the tissue. A short refractory period is a substrate for AF. Vulnerability to induced AF is assessed by sequential single, double and triple extrastimuli down to refractory period. Refractory period and vulnerability to AF are the primary end-points to be assessed across multiple surgeries, allowing comparison of the baseline state, vs a three-week postoperative state before and after administration of nanoformulated therapy to GP. High frequency subthreshold stimulation at 20Hz, at an output that does not capture atrium, is performed at the sites of GP. A positive response is defined by a change in heart rate of 5% or more after 10 sec of stimulation. We will be using Bloom electrical stimulator. Programmed atrial stimulation will not result in comparable high ventricular rates because of AV node protection. The high frequency stimulation of GPs is a subthreshold stimulation meant to stimulate autonomic nerves, without stimulating the heart. In our experience (Tan et al Circulation 2009), we expect any AF to be induced will be non-sustained (lasting a matter of seconds to minutes only). If AF does not self terminate, we will perform burst overdrive pacing to terminate AF. In our experience, this is always successful. Thus, no animal will be left in AF at the end of the procedure. Each EPS typically lasts 30 minutes.
Following this EP study, we will switch back to isoflurane maintenance. Nanoparticles will be injected into the left GP. After a 45 minute wait to allow effect of the nanoparticles to take effect, an EPS will be repeated. The left thoracotomy will be closed.

For dogs that undergo injection of 4 GP, requiring them right sided GP injection, a limited right thoracotomy will need to be performed. The dog will be flipped to the other side, re-prepped, and a right thoracotomy in the 5th intercostal space performed. Again, a limited thoracotomy will be performed as per left side. Because this side has not had prior surgery, we do not expected adhesions to be a problem. An EPS will be performed to induce AF. The right sided GP will be injected with nanoparticles. An EPS will be repeated. Then the thoracotomy will be closed and the animals recovered.

Dogs that undergo right thoracotomy only, the same EP study will be performed. However, the sites of stimulation will be on the right side (i.e., right atrial appendage, and left atrium adjacent to the right sided GP). High frequency stimulation will be performed, only from the right sided GP, which are adjacent to the right superior and inferior pulmonary veins. And only right sided GP will be injected with therapy. And thereafter, EPS will be repeated from those same sites.

In Group 1 (N=5), 4 GPs will be injected with calcium chloride alone (without nanoparticles). In Group 2a (N=5), all 4 GPs will be injected with nano-formulated calcium chloride In Group 2b (N=5), two left or right sided GPs will be injected with nano-formulated calcium chloride. In Group 3 (N=5), all 4 GPs will be injected with nano-formulated botox. AF induction will be performed before AND 1 hour after GP injection. AF induction before GP injection will serve as internal negative control (with the animal being a postoperative state after first surgery). Animals are allowed to recover on the ventilator with 100% oxygen until swallowing reflex is noted. The incision will be sprayed with a Vetericyn gel to promote wound healing. The endotracheal tube is removed and the canine is moved to a recovery cage with blankets and a warming pad. Once sternal, they receive meloxicam 0.2mg/kg IM.
The canine remains in the post-operative recovery cage until the following morning when they are moved to the standard chain link run with padding and blankets. They are given buprenorphine 0.01-0.02 mg/kg IM twice a day for three days for analgesia. Carprofen (2mg/kg PO) can be given for 1-3

days if pain is noted after the buprenorphine regimen is completed. Famotidine 0.5-1.0 mg/kg PO or IV can be given as needed for nausea or appetite stimulant (metoclopramide 0.2-0.5 mg/kg IV or bismuth subsalicylate 262 mg PO, can be given if needed). Some canines are too active post operatively which prevents incision healing. In this case, they are given Diazepam 0.2-2mg/kg IM or PO twice a day as needed. Cefpodoxime 5mg/kg PO is given once a day for 10 days to prevent wound infection. If there is ongoing concern for wound infection while receiving Cefpodoxime (Baytril 5-20mg/kg PO can be given once a day for 10 days). IV Fluids are available with or without 5% dextrose if an animal has not resumed normal eating habits within 2 days. Canine weights will be observed and recorded every other day while on antibiotics and then twice a weekly thereafter.
Following second surgery, animals will undergo 1 week of NA recordings.

Surgery 3 ►
The canine is pre-anesthetized with Acepromazine 0.05-0.1mg/kg approximately 1 hour before surgery. They are anesthetized with Brevital 6-10 mg/kg IV (Pentobarbital 30mg/kg IV can be given if Brevital is unavailable) to effect to allow for intubation with a cuffed endotracheal tube. The endotracheal tube is then connected to a vaporizer and respirator for isoflurane induction and mechanical ventilation. Isoflurane 1-3 % mixed with oxygen is used for surgical plane of anesthesia throughout the surgery unless otherwise described. Heart rate, blood pressure and temperature will be recorded every 15 minutes. Average heart rate for the dogs under anesthesia is 85-100 bpm. If heart rate increases by more than 5bpm from the average to this point, isoflurane may be temporarily increase by .5% (no more than 3% during surgery). Each dog will be weighed before surgery to determine the maximum dose of anesthetic that is allowed to prevent them from overdose. A left thoracotomy will be performed. Basic electrophysiologic studies will be performed in vivo, including measurement of effective refractory period, monophasic action potential and standard programmed stimulation protocols, including measurement of atrial effective refractory period. Blood will be drawn (less than 10 mls) from the aorta for norepinephrine levels.
After this initial period, we may switch from isoflurane maintenance to pentobarbital. If isoflurane inhibits the nerve activity we will give 5mg/kg of pentobarbital at this time prior to suspension of isoflurane and the heart rate will be monitored closely. Average heart rate for the dogs under anesthesia is 85-100bpm. If heart rate increases by more than 5bpm from the average to this point, small doses of pentobarbital (2mg/kg) will be administered to effect. Each dog will be weighed before surgery to determine the maximum dose of Pentobarbital that is allowed to prevent them from overdose. Once the nerve recording is finished, isoflurane will be resumed until end of surgery. Isoflurane has a nature of suppressing nerve activity and does so more than pentobarbital. For this reason, isoflurane is removed. If the maximum dose of pentobarbital is reached during surgery, isoflurane must be resumed and the EP study completed under the effects of pentobarbital.
We will perform EP studies to induce AF. The left thoracotomy will be partially closed using clamps. A right thoracotomy will be performed an EP study repeated to induce AF. Blood will be collected from coronary sinus as well as aorta by direct sampling. The animal will then be euthanized by exsanguination under anesthesia.

Tissues will be collected and preserved in 4% formaldehyde for 1 hour, before being stored in 70% alcohol. Additional tissue will be snap frozen in liquid nitrogen and stored for histopathology and molecular biology.

Surgery 4 ► **<u>Wound revision</u>**. Canines will on occasion be subject to surgical wound dehiscence. These animals will be returned to the OR for repair of the dehiscent wound, and drainage of any infectious or noninfectious fluid collections. . The canine is pre-anesthetized with Acepromazine 0.05-0.1mg/kg approximately 1 hour before surgery and given 2mg/kg Carprofen for analgesia. They are anesthetized with Brevital 6-10 mg/kg IV (Pentobarbital 30mg/kg IV can be given is Brevital is unavailable) to effect and placed on a mask for isoflurane induction. Isoflurane 2-4 % mixed with oxygen is used for surgical plane of anesthesia throughout the surgery unless otherwise described. Heart rate, blood pressure and

temperature will be recorded every 15 minutes. The procedure will be less than 30 minutes. The animal will recover in the post operative recovery cage with a warming pad until they can walk to their run. Vetericyn spray will be used to promote wound healing. They will be given Meloxicam 0.2 mg/kg IM after surgery and Cefpodoxime 5mg/kg PO for 10 days

3. **Personnel.** Complete the table below for each individual who will be involved in any of the surgeries on this protocol.

Name	Surgery #(s) (see Item 1)	Role in Surgery			
		Surgeon	Assistant	Manage Anesthesia	Other (describe)
[redacted]	1,2,3,4	(X)	( )	( )	( )
[redacted]	1,2,3,4	( )	(X)	(X)	( )
[redacted]	1,2,3,4	( )	(X)	(X)	( )
	1,2,3,4	( )	(X)	(X)	( )

4. **Location of surgery.** Complete the table below for each location where surgery on this protocol will be performed.

Building	Room Number	Surgery #(s) (see Item 1)	Type of Space		
			Dedicated Surgical Facility	Other Dedicated Surgical Space	Other Space not Dedicated to Surgery
[redacted]	[redacted]	1,2,3,4	(X)	( )*	( )*
			( )	(*)	( )*
			( )	( )*	( )*
			( )	( )*	( )*

*For each space that is not in a dedicated surgical facility, provide the justification for using this space for surgery on this protocol
►

5. **Pre-operative protocol.**

   a. **Pre-operative procedures.** Complete the table below for each pre-operative procedure that will be performed to prepare the animal(s) for surgery.

Surgery #(s) (see Item 1)	Fast (Specify Duration)	Withhold Water (Specify Duration)	Place Intravenous Catheter(s) (Specify Site(s))	Other – Describe
1	(X) – **12 Hours**	( ) --	( ) -- **brachial v**	( ) --
2	(X) – **12 Hours**	( ) --	( ) -- **brachial v**	( ) --
3	(X) – **12 Hours**	( ) --	( ) -- **brachial v**	( ) --
4	(X) --	( ) --	( ) -- **brachial v**	( ) --

b. **Pre-operative medications.** Complete the table below. Include agent(s) for induction of anesthesia, as well as any other pre-treatments that will be administered prior to preparation of the surgical site on the animal.

Agent	Surgery #(s) (see Item 1)	Dose (mg/kg) & volume (ml)	Route of administration	Frequency of administration (e.g., times/day)	Pre-operative period of treatment (e.g., immediate, or # of days)
Buprenorphine	1,2,	0.01-0.02 mg/kg	IM	Once	1 hour before
Acepromazine	1,2,3, 4	0.05-0.1mg/kg	Oral	Once	1-2 hours before
Pentobarbital	1,2,3,4	Up to 30mg/kg to effect	IV	Once, if brevital not available	Immediate
Brevital	1,2,3, 4	6-10 mg /kg	IV	Once	Immediate
Famotidine	1,2	0.5-1.0 mg/kg	Oral	Once	1 hour before
Carprofen	4	2mg/kg	Oral	Once	1 hour before

c. **Pre-operative preparation of the surgical site.** For each surgery, identify each surgical site on the animals, and describe how it will be prepared prior to surgery.

Surgery 1 ► **Hair to be clipped over the left thorax extending up to the neck and down to mid thorax (T7-8). Hair also to be clipped in the left flank. Surgery sites prepped with betadine. All survival procedures will be done using sterile techniques and surgical drapes.**

Surgery 2 ► **Hair to be clipped over the left thorax extending up to the neck and down to mid thorax (T7-8). Hair also to be clipped in the left flank. Hair clipping will be performed in surgical prep room. The animal will be transferred to the OR surgical suite. Surgery sites prepped with betadine. All survival procedures will be done using sterile techniques and surgical drapes.**

Surgery 3 ► **Hair to be clipped over the left thorax extending up to the neck and down to mid thorax (T7-8).**

Surgery 4 ► **Hair to be clipped over the incision in need of repair. Surgery sites prepped with betadine. All survival procedures will be done using sterile techniques and surgical drapes.**

6. **Intra-operative management.**

a. **Intra-operative medications.** Complete the table below for each agent that will be administered to the animal during surgery.

Agent	Paralytic*	Surgery #(s) (see Item 1)	Dose (mg/kg) & volume (ml)	Route of administration	Frequency of dosing
Isoflurane	( )*	1,2,3,4	1-4%	inhalation	Continous
Pentobarbital	( )*	1,2,3,4	Up to 30mg/kg to effect	IV	Once if needed
Brevital	( )*	1,2,3,4	6-10 mg/kg to effect	IV	Once if needed
Epinephrine	( )*	1	Low dose (0.01 mg/kg) every 3–5 min early; high dose (0.1 mg/kg) after prolonged; 1 ml	IV	if needed during VF
Amiodarone	( )*	1,	7 mg/kgbolus, repeated every 5 minutes as necessary	IV	if needed for VF

* For each agent shown above as a paralytic, explain why its use is necessary, and describe how the animals will be monitored to ensure that the depth of anesthesia is sufficient to prevent pain.
►

b. **Intra-operative physical support.** For each surgery, describe any physical support that will be provided for the animals during surgery (e.g., warming, cushioning, etc.).
►

c. **Intra-operative monitoring.** Describe the methods that will be used to monitor and respond to changes in the state of anesthesia and the general well-being of the animal during surgery.
►
The level of anesthesia will be continuously monitored by observation of arm cuff pressure, heart rate, and oxygen saturations every 15 minutes throughout surgery. Any increase in pressure or heart rate in response to surgical stimuli will be interpreted as indicative of inadequate anesthesia and supplemental

doses will be given. In addition, corneal, palpebral, and toe-pinch responses will be monitored every 15 minutes and supplemental anesthesia given as needed.
In order to most accurately view autonomic nerve activity during an EP studies isoflurane must be turned off for a period of 30-45 minutes. During this time Pentobarbital (5mg/kg) is given as follows to keep the animal in a surgical plane of anesthesia. The heart rate will be monitored closely. Average heart rate for the dogs under anesthesia is 85-100bpm. If heart rate increases by more than 5bpm from the average to this point, small doses of pentobarbital (2mg/kg) will be administered to effect.Each dog will be weighed before surgery to determine the maximum dose of pentobarbital (30mg/kg) that is allowed to prevent them from overdose. Once the nerve recording is finished, isoflurane will be resumed until end of surgery. Isoflurane has a nature of suppressing nerve activity and does so more than pentobarbital. For this reason, isoflurane is removed. If the maximum dose of pentobarbital is reached during surgery, isoflurane must be resumed and the EP study completed under the effects of isoflurane.

7. **Survival surgery considerations.** For each survival surgical procedure indicated in Item 1 and described in Item 2, complete Items 7.a. – 7.g.

   a. Complete the table below for each survival surgery listed in Item 1, above.

Surgery # (see Item 1)	Survival Period	Measures for Maintaining Sterility							
		Sterile Instruments	Surgical Cap	Sterile Gloves	Surgical Scrub	Sterile Drapes	Sterile Gown	Face Mask	Other*
1	6-8 weeks	(X)	(X)	(X)	(X)	(X)	(X)	(X)	( )*
2	2-4 weeks	(X)	(X)	(X)	(X)	(X)	(X)	(X)	( )*
4	6-8 weeks	( )	( )	( )	( )	( )	( )	( )	( )*
		( )	( )	( )	( )	( )	( )	( )	( )*

   * Describe any “other” measures to be taken to maintain sterility during surgery.
   ► **All surgeons and assistants will scrub hands with betadine before changing into sterile PPE.**

   b. For each surgery, describe the immediate post-operative support to be provided to the animals.

   For surgeries 1, 2, and 4: [redacted] **. Once extubated, the animal will be monitored until they are 2 hours post sternal. Acceptable heart rate parameters to leave the animal once sternal are 70- 160 bpm. Acceptable blood pressure parameters to leave the animal once sternal are systolic 90-160 mm of Hg and diastolic 50- 100 mm of Hg and respiration rate of <30.**

   c. Post-operative analgesia. Complete the table below for each surgery listed in item 1, above.

Surgery # (see Item 1)	Agent*	Dose (mg/kg) & Volume (ml)	Route of Administration	Frequency of Dosing (e.g., times/day)	Period of treatment (e.g. days)
1,2.	Buprenorphine	0.01-0.02mg/kg	IM	SID, Q8-12 hrs	3 days
1,2,4	Carprofen	2mg/kg	Oral	SID as needed	7 days
1,2,4	Meloxicam	0.2mg/kg	IM or SQ	Given once sternal after surgery and then SID as alternative to Carprofen	As needed

*For each surgery for which NO post-operative analgesic will be provided, enter "none" in the "Agent" column, and explain here why this is justified:
►

d. Other post-operative medications. Complete the following table to describe all other medications that will be administered as part of post-operative care.

Surgery # (see Item 1)	Medication	Dose (mg/kg) & Volume (ml)	Route of Administration	Frequency of dosing (e.g. times/day)	Period of treatment (e.g. days)
1,2,4	Diazepam	0.5-2mg/kg	Oral or IM	SID if needed	PRN
1,2,4	Famotidine	0.5-1.0 mg/kg	oral	SID	As needed
1,2,4	Cefpodoxime	5mgkg	Oral	SID,	10 days
1,2,4	Baytril	6-10mgkg	Oral	SID	7-10 days

e. Post-operative monitoring. <u>After-hours contact information for the personnel listed must be provided to the veterinary staff for use in case of an emergency</u>.

(1) Immediate post-operative monitoring

Surgery # (see Item 1)	Frequency of Monitoring	Duration at this Frequency	Name(s) of Responsible Individual(s)
1	Continuously	Until Sternal	[redacted]
2	Continuously	Until Sternal	[redacted]
4	Continuously	Until Sternal	[redacted]

(2) Post-operative monitoring after the immediate post-operative period

Surgery # (see Item 1)	Frequency of Monitoring	Duration at this Frequency	Name(s) of Responsible Individual(s)
1	6-12 hours	3-4 Days	[redacted]
2	6-12 hours	3-4 Days	[redacted]
4	6-12 hours	2-4 days	[redacted]

f. Post-operative consequences and complications.

(1) For each surgery, describe any common or expected post-operative consequences or complications that may arise and what will be done to address them.

Surgery 1 ►
During the surgical procedure, internal bleeding may occur due to cardiac or vessel laceration (lung or cardiac vessel). The PIs have learned a technique from a Cardiothoracic surgeon to repair lung lacerations but this still may not be survivable. If bleeding from any location becomes uncontrollable or not repairable, the animal will be euthanized by exsanguination under anesthesia.
During thoracotomy, fracturing the ribs is possible. If this does occur, the veterinarian will be notified immediately and analgesics will be continued for 7 days. Pneumothorax (inadequate seal of thoracotomy incision) can also occur immediately post operatively. This will require an emergency surgery to reclose the incision. Additional complications may include damage to structures in the surgical field such as pulmonary laceration resulting in hypoxia, pneumothorax, hemothorax, or pulmonary vascular laceration resulting in hypoxia. During suturing of electrodes to the atrium, this might cause laceration of the left atrium and pulmonary veins which could lead to uncontrollable bleeding. The phrenic nerve may become damaged during surgery leading to diaphragmatic paralysis. If the animal struggles to breathe once ventilation is stopped, the veterinarian will be consulted and the animal euthanized if this is not repairable.

Post operatively, the major concern is pain which can be treated effectively with analgesics. Animals that remain anorexic or with reduced appetite will be given IV or SQ fluids and treated with anti emetics. Hypertension may occur as a consequence of pain however, we only expect a mild degree of hypertension which is asymptomatic. We have never observed and do not expect severe malignant hypertension (BP >200/100mmHg). Severe hypertension can present with fatigue and neurologic impairment. If present, it can be treated with antihypertensives such as clonidine or hydralazine (as listed in medications list). Milder asymptomatic hypertension will be treated by analgesics (XX).

Post operative Infections may occur at the incision site. Cefpodoxime will be given as standard and Baytril will be given as an alternative if infection post op does occur or continues.

Surgery 2 ► Surgery 2 is a second survival thoracotomy. This is a pilot study in the sense that a survival thoracotomy had never been previously attempted, and second injection of nanoparticles had never been attempted. Thus, we expect increased risk of bleeding and collateral damage to tissues within the surgical field, and there may be additional complications that may not be listed below. The potential risks are:

1. Vascular injury – major blood vessels including pulmonary blood vessels may be

injured and result in excessive bleeding. If this cannot be hemostased, the animal will be euthanized.

2. Neural injury – there may be excessive bradycardia or tachycardia following denervation. In our experience we have observed differences in heart rate, but not enough to be symptomatic or to result in changes in blood pressure. Additionally, injury to the phrenic nerve can result in diaphragmatic paralysis, which may manifest as hypoxia due to unilateral lung atelectasis.
3. Ventricular fibrillation. Inadvertent injection into the ventricle (very unlikely) during the vulnerable period of cardiac repolarization may result in VF and animal death in case of failure of resuscitation. This is very unlikely as the GPs are in the atria rather than ventricles.
4. Pain from bilateral thoracotomy. Limited incisions will be performed. We will limit the incisions to about 5 cm. We will avoid reincising the same intercostal space as first surgery. Pain will be controlled with analgesics but should they not be sufficient, the veterinarian will be consulted for additional analgesia plans or euthanasia.
5. Wound Infection. Presence of bilateral thoracotomy increases the chances of wound infections. However we will treat this accordingly as above.
6. Nonsustained AF may be induced by programmed stimulation. We do not expect that AF will sustain beyond the duration of the surgery. We expect AF to last seconds, maybe minutes, if any.
7. Injury to lung and heart resulting in excessive and fatal bleeding and hypoxia from lung damage may result from release of adhesions, thus, damaging collateral structures. Adhesions are expected between lung and inner chest wall, lung and pericardium, pericardium and heart. Adhesions will be released as carefully as possible, and as minimally as possible. Where possible blunt dissection will be used. These injuries may be hidden because bleeding occurs internally into lung, thus we will closely monitor oxygen saturations and vitals postoperatively.
8. During injection of nanoparticles into epicardial ganglionated plexi, a micrographic needle is used to inject into epicardial fat pad and avoid intracardiac injection. Intracardiac injection might result in cardiac laceration, which might cause fatal bleeding as the wall of the atrial and pulmonary vein is thin and easily lacerated.
9.

Surgery 3 ►terminal surgery

Surgery 4 ►

Post-operative infection or intraoperative bleeding are the main concerns. If bleeding from any location becomes uncontrollable or not repairable, the animal will be euthanized by pentobarbital overdose. Post operatively, the major concern is pain which can be treated effectively with analgesics. Animals that remain anorexic or with reduced appetite will be given IV or SQ fluids and treated with anti-emetics (metoclopramide and famotidine)

(2) List the criteria for euthanasia related specifically to post-operative complications:

Surgery 1 ► Major nonsurvivable postoperative complications are described above. Any animal in pain or distress for longer than 72 hours that cannot be adequately treated with analgesics will be euthanized. Signs of distress and pain will suspected in the presence of weight loss (12% of body weight), lethargy, limping, vocalizing, excessive licking for over 2-3 days and even aggression. Wound infection not cured by antibiotics, pneumothorax, and pulmonary laceration resulting in hypoxia and respiratory distress symptoms, severe uncontrollable hypertension. Weights and temperatures will be monitored.

Surgery 2 ► Major nonsurvivable postoperative complications as described above. Any animal in pain or distress for longer than 72 hours that cannot be adequately treated with analgesics will be euthanized. Signs of distress and pain will suspected in the presence of weight loss (10% of body weight), lethargy, limping, vocalizing, excessive licking for over 2-3 days and even aggression. Wound infection not cured by antibiotics, pneumothorax, respiratory distress symptoms, and hypertension**.**

Surgery 3 ►terminal surgery

Surgery 4-The major concern for this minor procedure is pain and this will be treated with analgesics. Standard wound care will be provided. Animals who are slow to resume normal eating patterns will be given fluids either IV or SC and anti-emetics

(3) In case an emergency medical situation arises and none of the research personnel on the ACORP can be reached, identify any drugs or classes of drugs that should be avoided because of the scientific requirements of the project. (If the condition of the animal requires one of these drugs, the animal will be euthanatized instead.)
► **NONE**

g. Maintenance of post-surgical medical records. Complete the table below for each surgery, specifying where the records will held, and identifying at least one individual who will be assigned to maintain accurate, daily, written post-surgical medical records. Indicate whether the named individuals are research personnel involved in this project, or members of the veterinary staff.

Surgery # (see Item 1)	Location of Records	Name(s) of Individual(s) Responsible for Maintaining Written Records	Research Personnel	Veterinary Staff
1	[redacted]	[redacted]	(X)	( )
2	[redacted]	[redacted]	(X)	( )
3	[redacted]	[redacted]	(X)	( )
4	[redacted]	[redacted]	(X)	( )

8. **Certification.** The PI must sign the certification statement in Item Z.5 of the main body of the ACORP.

ACORP APPENDIX 6
SPECIAL HUSBANDRY AND PROCEDURES
VERSION 4

See ACORP App. 6 Instructions, for more detailed explanations of the information requested.

1. **Description of Procedures.** Complete the table below for each procedure listed in Item V of the main body of the ACORP that is not detailed in a SOP or in another item or Appendix of the ACORP. For each special procedure, check <u>all</u> features that apply.

Special Procedure	Features

Number	Brief Description	Husbandry	Restraint	Noxious Stimuli	Exercise	Behavioral Conditioning	Irradiation	Imaging	Other**
1	Nerve recording. Cage cleaning and letting of dogs out of cage will need to be done one dog at a time, so as to avoid data crosstalk between cages (receivers meant for another dog picking up data from this dog as the dog runs around the room). Also, times will have to be pre-specified so the P.I. can expect when data drop out or potential cross talk occurs.	**(√)**	**( )**	**( )**	**( )**	**( )**	**( )**	**( )**	**( )**
2	Drug Challenge	(X)	()	( )	( )	( )	( )	( )	( )

*Husbandry refers to all aspects of care related to the maintenance of the animals, including (but not limited to) provision of an appropriate diet, access to water, control of environmental conditions, and the selection of primary and secondary enclosures.

**Describe any "Other" features that are involved.
►

a. Provide a complete description of each special procedure listed above, including the duration of the procedure, how frequently it will be repeated in any one animal, and any effects it is expected to have on the animal:

Special Procedure 1 ► Typically cage cleaning is performed daily with dogs let out of the cage in random fashion. However, because the receivers installed in each cage has the capability of picking up signals from any dog that is in close proximity to it, letting dogs out all at the same time has the ability to cause data cross talk, thus invalidating the data for that period of time when the dogs are out of the cage. For this reason, the dogs with transmitters in the same room have to be let out individually, returned to the cage, before another dog with a transmitter be let out of its cage. The times need to be documented as well so the P.I. can expect when data drop out or potential cross talk occurs. In general cleaning occurs in the mornings. Cleaning times will be posted clearly on the dog runs and communicated to the VMU supervisor and caretakers.

Special Procedure 2 ► Drug Challenge. In order to understand and validate recordings in the renal nerve/ganglia and relationship between stellate and vagal nerve, we plan to pharmacologically stimulate autonomic nerves by administration of short-acting intravenous vasoactive drugs (clonidine or phenylephrine) during the chronic monitoring phase.

These drugs used are ones commonly used in clinical practice in humans but have been also used in canines. They are all short-acting drugs whose half-lives do not exceed 12 hours when given orally. Therefore, when administered IV, their effects peak within minutes and half-lives usually less than 4 hours as described below:

IV clonidine (10 µg/kg) is an alfa-2 agonist which will suppress central sympathetic nerve discharge by acting on pre-synaptic alfa-2 receptors in sympathetic nerve terminals. IV Clonidine peaks within an hour and has a plasma half-life of 2-3 hours. Blood pressure and heart rate are expected to drop but the doses used have been reported in the literature [Cavero, Br. J Pharmacol 1980; 70:269]. We expect that the effects are transient and will not have long term sequelae.

There is a slight risk that ventricular tachycardia could occur. This has been observed to happen in denervated animals in another protocol, however this is not a problem that is anticipated in these animals. To combat this, the animal will be monitored for 60 minutes post administration. If sustained VT is observed on the DSI recording for more than 2 minutes, the animal will be administered Amiodarone (7mg/kg IV, every 5 minutes as needed). If hypotension occurs, we will administer fluids. Because this medication is transient, we do not expect sudden cardiac death to occur. We have not observed any evidence of dramatic hypotension as a result of this medication in either two animals.

IV phenylephrine 0.01 mg/kg. Phenylephrine is a vasopressor used to increase BP. This will be given as an IV bolus. The increase in BP will suppress sympathetic nerve activity and potentiate vagal nerve activity. We expect that the effects are transient and will not have long term sequelae.
[Moise, N., Moon, P. F., Flahive, W. J., Brittain, D., Pride, H., Lewis, B. A., ... & Gilmour, R. F. (1996). Phenylephrine-Induced Ventricular Arrhythmias in Dogs with Inherited Sudden Death. *Journal of cardiovascular electrophysiology*, *7*(3), 217-230.] [Varma S, Circulation Research 1960;8:1182].
T

The animals will be challenged twice with each of the above drugs. Once approximately two weeks following recovery from survival surgery. The second time will be the week following the second survival surgery, right before the terminal surgery.

We will *maintain a log* of blood pressure readings, heart rate, time of administration and physical characteristics during the monitoring phase and keep this information in the animals folder for review.

b. Explain why each of these special procedures is necessary:

Special Procedure 1 ► As soon as PVC software patch is enabled, DSI radiotelemetry device will be turned ON to record vagal and renal autonomic nerve activity and monitor the relationship between cardiac and the development of PVC-induced CM. However, because the receivers installed in each cage has the capability of picking up signals from any dog that is in close proximity to it, letting dogs out all at the same time has the ability to cause data cross talk, thus invalidating the data for that period of time when the dogs are out of the cage.

Special Procedure 2 ► The purpose of the IV pharmacological challenge is to characterize the behavior of the renal sympathetic nerve recordings and its correlation with cardiac sympathovagal nerves at baseline and after VT induction with subsequent GP injection. The interventions are designed to perturb blood pressure and consequently baroreflexes, which will stimulate or suppress cardiac sympathovagal and/or renal sympathetic nerves, or suppress central sympathetic outflow.

2. **Personnel.** Complete the table below for each special procedure listed in Item 1, above. Identify the individual(s) who will be responsible for carrying out the procedures, and those who will be responsible for monitoring the condition of the animals during and after the procedures. After-hours contact information for the personnel listed must be provided to the veterinary staff for use in case of an emergency.

Procedure Number (see Item 1)	Responsible Individual(s)	
	Carrying Out Procedure	Monitoring the Animals
1	Cage cleaning will be performed by VMU husbandry staff.	Veterinarian, animal caretakers and protocol staff
2	[redacted]	[redacted]

3		

3. **Potential Pain or Distress.** Complete the table below for each special procedure identified in Item 1, above, indicating for each procedure, whether potential pain and/or distress is expected, and, if so, describing the potential pain and/or distress and indicating whether any measures are to be taken to prevent or alleviate it.

Procedure Number (see Item 1)	Expected Potential Pain and/or Distress			
	No	Yes		
		Description	To Be Relieved	Not to Be Relieved
1	(√)		( )[a]	( )[b]
2	( )	There is a slight risk that ventricular tachycardia could occur. This has been observed to happen in denervated animals in another protocol, however this is not a problem that is anticipated in these animals. To combat this, the animal will be monitored for 60 minutes post administration. If sustained VT is observed on the DSI recording for more than 2 minutes, the animal will be administered Amiodarone (7mg/kg IV, every 5 minutes as needed). If hypotension occurs, we will administer fluids. Because this medication is transient, we do not expect sudden cardiac death to occur. We have not observed any evidence of dramatic hypotension as a result of this medication in either two animals.	(X)[a]	( )[b]

a. For each procedure for which potential pain and/or distress is expected, but <u>WILL be prevented or alleviated</u> by administration of the analgesic(s) or stress-relieving agents, complete the table below:

Procedure Number (see Item 1)	Agent	Dose (mg/kg) & vol (ml)	Route of admin	Freq of admin (times/day)	Duration of admin (days post-procedure)
1					
2	Amiodarone	7mg/kg	Bolus IV	Every 5 minutes as needed	Day of
3					

Describe any non-pharmacological measures to be taken to address the potential pain and/or distress:

Special Procedure 1 ►

Special Procedure 2 ► The medications given have not been observed to cause any major side effects. As such we have not had to treat it.

Special Procedure 3 ►

Special Procedure 4 ►

b. For each procedure for which potential pain and/or distress is expected and will NOT be prevented or alleviated, provide the scientific justification for this:

Special Procedure 1 ►

Special Procedure 2 ► We have not observed any major side effects with IV pharmacologic challenge that require therapy. Generally, if nausea is present secondary to the medications, they resolve within an hour, which is the approximate half life of IV administered drugs. We have observed transient unsustained arrhythmias (PVCs or unsustained VTs lasting <10 beats) in denervated animalms that do not require any therapy. If necessary, IV amiodarone 7mg/kg can be given.

Special Procedure 3 ►

Special Procedure 4 ►

4. **Monitoring.** Describe how the condition of the animals will be monitored during and after each of the special procedures, and list the criteria that will be used to determine when individual animals will be removed from groups undergoing these procedures, because of pain or distress (see ACORP App. 6 Instructions, for details):

Procedure Number (see Item 1)	Monitoring Methods	Endpoint Criteria
1	2 DSI telemetry devices are attached to dog runs to monitor routine, daily behavior	At the end of study, just before final surgery, telemetry devices will be turned off
2	Present with dog and blood pressure monitoring during procedure and close observation throughout full recovery. The animal will be observed for 60 minutes following administration to combat spontaneous VT.	Drug challenge would not be obtained in those animals with any kind of distress / recent wound or surgical procedures.
3		

Secondary Just-In-Time ACORP Review

PI	STATION	CYCLE	APPLICATION TITLE
[redacted]	Richmond, VA 652	Commonwealth of Virginia	Nanoparticle Injection into Ganglionated Neural Plexi to Prevent Atrial Fibrillation - 02235

	SCORE	DESCRIPTION	ACTION NEEDED BY IACUC
○	0	No concerns noted. Any comments provided are for information only.	*None*. No further correspondence with the CVMO is needed; the ACORP(s) is(are) cleared and represent(s) no bar to funding the application.
●	1	Some concerns noted.	*The IACUC must review the **level 1** concerns listed below and decide what response is needed. This action must be documented in the IACUC minutes and the changes required by the IACUC must be incorporated into the ACORP(s).* No further correspondence with the CVMO is needed; the ACORP(s) is(are) cleared and represent(s) no bar to funding the application.
○	2	Concerns are noted that must be addressed by the local IACUC and PI before funding can occur, but work described in the ACORP(s) may continue.	*A response to each of the **level 2** concerns noted below must be reviewed and cleared by the CVMO* before funding can be released. Upload the following at https://vaww.gateway.research.va.gov: (1) a memo addressing the concerns, dated and signed by the PI, veterinarian, and IACUC Chair; and (2) (a) revised ACORP(s) approved by the IACUC. *The IACUC must review each of the **level 1** concerns listed and decide what response is needed. This action must be documented in the IACUC minutes and the changes required by the IACUC must be incorporated into the ACORP(s).*
○	3	Significant concerns are noted that must be addressed by the local IACUC and PI before funding can occur, and work described in the ACORP(s) listed below must cease immediately.	*A response to each of the **level 3** concerns listed below must be reviewed and cleared by the CVMO* before work can resume and funding can be released. (If unusual circumstances dictate that work should continue despite concerns, notify the CVMO immediately.) *A response to each of the **level 2** concerns noted below must be reviewed and cleared by the CVMO* before funding can be released. For **level 2 and 3** concerns, upload the following at https://vaww.gateway.research.va.gov : (1) a memo addressing the concerns, signed by the PI, veterinarian, and IACUC Chair; and (2) (a) revised ACORP(s) approved by the IACUC. *The IACUC must review each of the **level 1** concerns listed and decide what response is needed. This action must be documented in the IACUC minutes and the changes required by the IACUC must be incorporated*

(cont.)

			into the ACORP(s).

The ACORP for Dr. [redacted] has received an overall score of 1, which means that it is cleared and represents no bar to funding the application, although some concerns were raised, as shown below.

Please note that a separate score is shown for each of the individual concerns (shown in parentheses under the Item number to which each of the individual concerns refers), to assist you in interpreting the review. An explanation of each of the levels of concern is shown above, in the chart on the previous page. The IACUC must review each of the **level 1** concerns listed and decide what response is needed. This action must be documented in the IACUC minutes, and the changes required by the IACUC must be incorporated into the ACORP, but no further correspondence with the CVMO is needed.

In case of questions about this review, please contact Dr. [redacted], Assistant Chief Veterinary Medical Officer at [redacted] or [redacted].

REVIEWER FEEDBACK

ACORP Item number(s) (score)	Comments/Concerns
ACORP (dog)	This ACORP uses a canine model of induced atrial fibrillation (AF) to determine if nanoparticles can effectively be used to release neurosuppressants and prevent AF postoperatively. The investigator has provided a strong justification for research to identify methods that will more effectively manage AF than radiofrequency ablation. The investigator is commended for providing a clear rationale for the procedures proposed and particularly for bilateral thoracotomy, detailed procedural descriptions often supported with literature citations, well-defined humane endpoints and for being present during the immediate post-operative period to assist the research staff in managing the dogs as they recover from major surgery. The comments provided are informational only and are offered for the committee's consideration. An appendix to this review provides additional information for the IACUC's consideration. Note: Although, this protocol was well-written overall; several misspellings were identified, including but not necessarily limited to: alfa" for "alpha", "infeciton" for "infection," and "animalms" for "animals." Please correct.
Item C.2 (1)	The investigator proposes the following experimental groups: Group 1: injection of calcium chloride without nanoformulation into 2 left sided atrial ganglionic plexi (GP) Group 2: injection of nanoformulated calcium chloride into 4 GPs Group 2b: injection of nanoformulated calcium chloride into 2 GP Group 3: injection of nanoformulated botox into 4 GPs. For the botulinum toxin (botox) groups, why were the following not included: Group 4: injection of botox without nanoformulation in 2 left sided GP Group 5: injection of nanoformulated botox into 2 GP. Please address.

(cont.)

	If AF does not self-terminate, the investigator will perform burst overdrive pacing to terminate AF; please explain the burst overdrive pacing procedure.
Items C.2 and W (1)	Overall the justification is well-written; please consider also addressing the contribution of *in vitro* studies to this project and why non-mammalian studies are unacceptable. Why are only female dogs used?
Item C.2, Appendix 3 and Appendix 5 (1)	Item C.2 and Appendix 5 indicate that neurosuppressants or nanoparticle formulated neurosuppressants are injected into the GP once during surgery. However, in item 7.f of Appendix 5, the investigator states "This is a pilot study in the sense that a survival thoracotomy had never been previously attempted, and second injection of nanoparticles had never been attempted. Thus, we expect increased risk of bleeding and collateral damage to tissues within the surgical field, and there may be additional complications…" Please address this inconsistency in the protocol.
Item D (1)	VA Policy requires research training be renewed every two years.
Item J (1)	Terminal surgery is described in item C.2 and Appendix 5, please list this category D procedure in item J.
Item U (1)	If an overdose of pentobarbital is used, how is death confirmed? Terminal surgery concluding with exsanguination under anesthesia is noted in several ACORP items, please list this method of euthanasia in item U.
Appendix 5 (1)	In regard to the need for wound revision, wound dehiscence soon after surgery is often related to the sutures being tied too tightly, which compromises blood supply. In dogs, the sutures should be tightened (tied) just enough to appose the skin edges; the surgeon should be able to place the tip of a small hemostat beneath each interrupted suture. Skin breakdown over a subcutaneously implanted device is also usually related to pressure necrosis.

Appendix:

652[redacted]02235dogs20170718 secondary review 11-6-2016

Parts of this ACORP would benefit from being rewritten for clarity. Some justifications and explanations could be made stronger.

DETAILED COMMENTS:

Comment 1: General comment: please include a list of abbreviations.

Comment 2: Section B: This section would be stronger if the relevance to veterans' health was pointed out. Try putting something like this after the first paragraph:

(cont.)

The National Heart, Lung, and Blood Institute at NIH lists high blood pressure as a major risk factor for AF (see https://www.nhlbi.nih.gov/health/health-topics/topics/af/atrisk accessed 11/6/2017). A study published in 2016 showed that over 81% of Vietnam Veterans who sprayed herbicides (Agent Orange) over Vietnam have high blood pressure, putting them at higher risk for AF than other Veterans or the general population (see https://www.ncbi.nlm.nih.gov/pubmed/27820763 accessed 11/6/2017).

Comment 3: Section D: This section could be made stronger by pointing out more of the reasons dogs are used for this study. Try something like this:

Dogs are used in this study because they can get AF very much like human AF. Unfortunately, smaller animals such as rats and mice do not get AF much so they would not work for this study. The project also requires implanting a radiotelemetry device which is too large for animals such as rabbits and guinea pigs.

Our only options for this work are large animals such as dogs and pigs. Dogs are much more suitable for this work because the canine heart has a His-Purkinje system located in endocardium just like in the human heart. Pigs and other larger animals have a different anatomy, so work with them would not be as relevant to the human condition. Canine heart physiology has also been extensively studied over many years so a lot of information is already available that our study builds upon. Switching to another species such as pigs would require us to start over to some degree, running a lot of pig experiments to reach the point where we already are with dogs before we could even perform this particular study. This process would use many more pigs to get to that point than the 24 dogs required for this study.

Comment 4, section W1 table (literature search). This literature search would be strengthened by doing the following:

1) Include nanoparticle injection in the list of potentially painful or distressing procedures above the table.
2) Change how the search terms are run. Running all the potentially painful or distressing procedures together in a single search means only a paper that includes all of those search terms would be found. Run separate searches instead, since there may be papers that address individual procedures.

In the example below, the first row is a search for unnecessary duplication, focusing on this particular study. The rest are for potentially painful or distressing procedures. Each if these searches brings up less than 30 papers, using the ALTBIB website run by NIH

(cont.)

https://toxnet.nlm.nih.gov/altbib.html for the years 2000 – present. (This website works with Google Chrome, but not with Internet Explorer)

Name of the database	Date of search	Period of years covered by the search	Potentially painful or distressing procedures addressed	Key words and/or search strategy used	Indicate which mandate each search addressed			
					Replacement of animals (item W.2)	Reduction in numbers of animals used (item W.3)	Refinement to minimize pain or distress (item W.4)	Lack of unnecessary duplication (item W.5)
PubMed	11/6/17	1966-2017	N/A	atrial fibrillation, nanoparticle	( )	( )	( )	(X)
PubMed using ALTBIB animal alternatives search strategy	11/6/17	2000-2017	Injecting nanoparticles into the ganglionic plexi	nanoparticle injection, ganglionic plexus	(X)	(X)	(X)	( )
PubMed using ALTBIB animal alternatives search strategy	11/6/17	2000-2017	electrical stimulation induced atrial fibrillation	electrical stimulation induced atrial fibrillation	(X)	(X)	(X)	( )
PubMed using ALTBIB animal alternatives search strategy	11/6/17	2000-2017	Thoracotomy for implanting the pacemaker and radiotelemetry device	thoracotomy, pacemaker implant	(X)	(X)	(X)	( )
PubMed using ALTBIB animal alternatives search strategy	11/6/17	2000-2017	Thoracotomy for injecting nanoparticles into the ganglionic plexi	thoracotomy, nanoparticle injection	(X)	(X)	(X)	( )
PubMed using ALTBIB animal alternativ	11/6/17	2000-2017	Blood draw from the aorta for norepinephrine analysis	blood draw, aorta	(X)	(X)	(X)	( )

(cont.)

es search strategy								
PubMed using ALTBIB animal alternatives search strategy	11/6/17	2000-2017	Blood draw from brachial or jugular veins	blood draw, (brachial OR jugular)	(X)	(X)	(X)	( )

Comment 5, section W2: (Replacement)

This section would be strengthened by including the following information:

a) **Please explain why this research cannot be done with *in vitro* methods.**

b) **Please explain if there are any computer models for AF, and if so why they cannot be used for this work.**

c) **The text states that "**Other large animals such as pigs are not as suitable for chronic instrumentation from a behavioral standpoint" . **Please provide some detail about the behavioral issues that make pigs and other large animals unsuitable for this work.**

Comment 6, section W3: (Reduction)

The answer provided in this ACORP would be strengthened by adding a sentence like this at the end:

"We ran a power calculation to determine the smallest number of animals needed for this study. See section C2b for details."

Comment 7, section W5 (lack of unnecessary duplication)

The answer provided in this ACORP is would be strengthened by including a discussion of the papers the literature search brought up and how this study does not unnecessarily duplicate them.

Try adding a sentence like this at the end:

Our literature search for "atrial fibrillation, nanoparticle" yielded only six papers. One was actually a tumor study, one was about anticoagulants, one was a study of the KCNE1 protein in cell membranes, one was studying a potassium channel, and one was actually an editorial. One paper

(cont.)

used nanoparticles given intravenously to treat AF. Our project will be directly targeting the nanoparticles to the ganglionic plexi to treat AF, which we think will be more effective and cause fewer side effects on other tissues.

(cont.)

Literature search 652 Richmond [redacted]

1) **How is this research relevant to Veterans health?**

This study looks at treating atrial fibrillation (AF) with nanoparticles of botulinum toxin or CaCl2 injected into atrial ganglionic plexi as an alternative to radiofrequency ablation.

The National Heart, Lung, and Blood Institute at NIH lists high blood pressure as a major risk factor for AF (see https://www.nhlbi.nih.gov/health/health-topics/topics/af/atrisk *accessed 3/11/18*). A study published in 2016 showed that over 81% of Vietnam Veterans who sprayed herbicides (Agent Orange) over Vietnam have high blood pressure, putting them at higher risk for AF than other Veterans or the general population (see https://www.ncbi.nlm.nih.gov/pubmed/27820763 accessed 3/11/18). Better treatments for AF would especially benefit this high-risk population of Veterans.

2) **Is this work <u>unnecessarily</u> duplicating work already documented in the literature?**

Name of the database	Date of search	Period of years covered by the search	Key words and/or search strategy used	How many papers were found?
PubMed	3/12/18	All available years	atrial fibrillation, nanoparticle	6

A PubMed search for "atrial fibrillation, nanoparticle" yielded only six papers. One was actually a tumor study, one was about anticoagulants, one was a study of the KCNE1 protein in cell membranes, one was studying a potassium channel, and one was actually an editorial. One paper used nanoparticles given intravenously to treat AF. The current project will be directly targeting the nanoparticles to the ganglionic plexi to treat AF, which should be more effective and cause fewer side effects on other tissues.

3) **Could this work be done in computer models or in vitro (tissue culture)?**

Name of the database	Date of search	Period of years covered by the search	Key words and/or search strategy used	How many papers were found?
ALTBIB Citations with <u>Animal Use Alternatives</u> as the main topic	3/12/18	All available years	atrial fibrillation, nanoparticle	0

An ALTBIB search for “alternatives to using animals” for this study yielded no papers at all. No computer models or in vitro models for this work were found.

4) Could it be done in non-mammals or in other mammals?

Name of the database	Date of search	Period of years covered by the search	Key words and/or search strategy used	How many papers were found?
ALTBIB animal alternatives search strategy - all citations	3/12/18	2000-present	atrial fibrillation, nanoparticle	0

An ALTBIB search for all citations for this study yielded no papers at all. This is a very new area and no other animal models have been established.

Small mammals such as rats, mice, guinea pigs and rabbits cannot be used for this project because the only technology available to deliver PVCs in a controlled fashion is through a special highly sophisticated (approximately 2 inches long, ¼ inch thick and 1.5 inch wide) electronic defibrillator / pacemaker that is too large to use in small animals. The radiotelemetry device is also large and will require internal implantation and observation for several months.

Dogs are used for two reasons: 1) Atrial fibrillation can be readily induced in dogs and 2) The group has been using dogs for over 25 years. In order for the new data to be comparable with the previously collected data, they need to continue to use dogs. Switching to another species would to some degree be starting over, and require many more animals than this study will use.

Although in principle pigs could be used, the electrophysiology of the pig heart differs from the human and canine heart in a significant way, specifically the endocardium and epicardium are activated simultaneously in the swine heart but not in the human or canine heart [Lelovas 2014], and this discordance in the human and dog heart can play a role in the development of cardiomyopathy. Additionally, dogs have a His-Purkinje system located in endocardium, very similar to the human heart, that pigs and other larger animals do not have [Newton 2004].

5) **Are the methods used the best available (least painful or distressing to the dogs)?**

Name of the database	Date of search	Period of years covered by the search	Potentially painful or distressing procedures addressed	Key words and/or search strategy used	How many papers were found?

ALTBIB animal alternatives search strategy - all citations	3/11/10	2000-2018	chronic PVC-induced left ventricular dysfunction	chronic PVC-induced left ventricular dysfunction	0
ALTBIB animal alternatives search strategy - all citations	3/11/10	2000-2018	thoracotomy	thoracotomy, "cardiac surgery"	5
ALTBIB animal alternatives search strategy - all citations	3/11/10	2000-2018	symptomatic congestive heart failure	"symptomatic congestive heart failure", canine	0

We ran multiple searches for better methods:

1) A search on ALTBIB for “chronic PVC-induced left ventricular dysfunction” yielded no papers.
2) A search on ALTBIB search for “thoracotomy, cardiac surgery" gave 5 papers. Two were on rats, one was one sheep, and one was on mice. The fifth paper was about transcatheter pulmonary valve replacement, which is very different from what we are doing in this study. We did not find refinements for thoracotomy in canines for our studies.
3) A search on ALTBIB for “symptomatic congestive heart failure, canine” yielded no papers.

This group has extensive experience in this surgical model of PVC-induced cardiomyopathy, a model that they designed. The method has been refined so the cardiomyopathy develops gradually without symptomatic congestive heart failure or signs of distress.

<u>The treadmill procedure is designed to minimize distress for the dogs:</u>

> Dogs allowed to them explore the exercise room and equipment until they have become comfortable with those surroundings. Presence of normal, relaxed behavior will signal that the dogs are ready for the next step, which is putting them on the treadmill while it’s turned off. This will occur in small steps, putting them on for seconds and then extending the time. Each positive reaction will be rewarded with treats to encourage the dogs’ learning process. When the dogs have become relaxed with the task of being on the still treadmill, they will next be put on the treadmill at its slowest speed, 0.5 mph. Two people will assist in this process; one person will hold the leash of the dog and stand in front of the treadmill offering rewards for positive behavior while the other will stand behind the animal making sure that it does not slide off of the machine or jump off of the sides. This person will also to help the dog move its feet until it begins to understand and be comfortable with the movement. The process will take as long as needed to have the dogs become comfortable with the treadmill**.**
>
> The treadmill workout will be done a total of 4 times in our study. The first 2 workouts will be performed 1-2 days apart at baseline about 2 weeks post-surgery after sutures have

been removed. The final 2 treadmills will occur 1-2 days apart at the end of the study before final surgery.

Each workout lasts 10 minutes, in which the dogs will complete 3 stages, each lasting 3 minutes. at the first stage is at 1.1 mph followed by three minutes at 2.3 mph, and then three minutes at 3.3 mph. (Normal human walking speed is about 3 mph).

<u>The procedures for echocardiograms, blood draws, etc., are also designed to minimize distress for the dogs:</u>

Non-surgical procedures such as echocardiograms, electrocardiography, pacemaker interrogation and blood draws will be performed in conscious dogs with minimal or no sedation. In order to achieve this, all animals will undergo training to lay or sit down still for 20-30 minutes during the procedures. We estimate that this training will take from 2-4 weeks. Methods used for training will consist mostly of repetition with rewards as the periods of lying or sitting still are gradually extended.

However, if an animal cannot be trained to sit or lie supine for 10 minutes for the echocardiogram, we will first attempt to mildly sedate the animal with Acepromazine (0.05-0.1mg/kg) given PO approximately 1 hour prior to the procedure. If this is unsuccessful we will have to perform echocardiogram under general anesthesia with endotracheal intubation. We will use Brevital (6-10mg/kg) IV to effect (or Pentobarbital 30mg/kg, if Brevital is not available). Animals will be intubated, mechanically ventilated and anesthetized with isoflurane 1-3%. After the echocardiogram, they will be allowed to recover from anesthesia in a post-operative recovery cage until able to walk to their kennel. No analgesics will be necessary due to the non-invasive nature of this procedure.