Published in final edited form as:

Heart Rhythm. 2019 April ; 16(4): 597–605. doi:10.1016/j.hrthm.2018.10.014.

Epicardial Injection of Nanoformulated Calcium into Cardiac Ganglionic Plexi Suppresses Autonomic Nerve Activity and Postoperative Atrial Fibrillation:

Nanomedicine Therapy for Post-Op Atrial Fibrillation

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Abstract

Background—Imbalanced activation of the cardiac autonomic nervous system (CANS) triggers postoperative atrial fibrillation (POAF). Neuronal calcium overload induces apoptosis. We hypothesize that epicardial injection of timed-release nanoformulated-CaCl₂ (nCaCl₂) into LA ganglionic plexi (GP) modulates autonomic function and suppresses POAF.

Objective—Determine whether nCaCl₂ GP therapy suppresses POAF.

Methods—We utilized a novel canine model of POAF with implanted radiotelemetry to record nerve activity (NA) from left stellate ganglion (SNA), vagus (VNA), and GP (GPNA). At week 3 (PO-3wk), nCaCl₂ (n=7) or vehicle-control (sham, n=3) was injected into left pulmonary vein (PV) GP (LGP), followed by right PV GP (RGP) at week 4. Atrial effective refractory period (AERP) and atrial fibrillation vulnerability (AFV) were assessed in-vivo. Resting and exercise NA and HR were assessed before and after LGP treatment.

Results—AERP decreased (p<0.0001) and AFV increased (p=0.008) at PO-3wk vs baseline. However, nCaCl₂-LGP-treatment reversed these changes and restored them to baseline after 1 week (p=0.04). Subsequent nCaCl₂-RGP-treatment reduced AFV further (p=0.03). In contrast, AFV increased (p=0.001) and AERP remained decreased (p=0.01) 1 week after sham-LGP

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Conflicts: Dr Dormer is a co-founder of Nanomed Targeting Systems (NTS) Inc.

treatment vs baseline. nCaCl₂-LGP-treatment reduced GPNA ($p<0.02$) and VNA ($p<0.05$) and increased SNA ($p<0.02$). Despite increased SNA, HR was decreased ($p<0.01$) with loss of HR-SNA correlation ($R=0.62$). After sham-LGP treatment, NA was unchanged and HR-SNA remained correlated ($R=0.95$). Histology confirmed nCaCl₂-GP co-localization, apoptosis and loss of immunoreactivity in nCaCl₂-treated soma.

Conclusion—Epicardial injection of nCaCl₂ into LA GP induced neuroapoptosis and modulated autonomic function. This reversed a postoperative reduction in AERP and suppressed POAF.

Keywords

postoperative atrial fibrillation; autonomics; cardiac surgery; neural modulation; chronic animal model

Introduction

Imbalanced activity in sympathetic and parasympathetic arms of the cardiac autonomic nervous system (CANS) triggers AF^{1,2} and POAF³. The post-cardiac surgery state is characterized by sympatho-excitatory surge along with inflammatory, metabolic and hemodynamic derangements that activate cholinergic neural elements of the heart³ and promote POAF.^{1,2} Therefore, targeted CANS modulation could be anti-arrhythmic against POAF.⁴⁻⁶ The CANS consists of intrinsic and extrinsic components, the intrinsic being a network of multiple ganglionated plexi (GP) and interneurons located within subepicardial fat pads including those adjacent to pulmonary veins.^{4,5} We hypothesize that CaCl₂, when encapsulated within timed-release nanoparticles (nCaCl₂) and injected epicardially into GP, will modulate GP function and suppress POAF vulnerability. Targeted GP therapy using a ubiquitous substance such as calcium, can be administered during open heart surgery, would advantageously avoid the myocardial damage caused by radiofrequency ablation and potentially induce sustained effects on CANS and POAF.

Calcium ions, though essential for cellular function, are excessively internalized by neurons when exposed to greater than 0.1mM in the presence of L-glutamate.⁶ L-glutamate binds neuronal NMDA receptors enhancing neuronal calcium internalization.⁶ The resulting increased intracellular calcium concentration triggers neuroapoptosis,⁶ providing persistent CANS modulation. We have previously formulated CaCl₂ with trace L-glutamate into polymeric nanoparticles consisting of poly-(lactide-co-glycolide)(PLGA)⁷ for burst followed by extended release after injection into GP and warming to body temperature. This enables specific targeting of GP and prolonged neural exposure to high calcium concentrations, while avoiding neighboring tissues. The goal of this study was to determine whether epicardial injection of nCaCl₂ into LA GP, inhibited their function and suppressed POAF, whilst being non-toxic to adjacent myocardium. To achieve these goals, we developed a novel canine model of POAF with sequential survival thoracotomies, which creates a substrate for AF due to successive postoperative injury and repair, and enabled chronic assessments of nCaCl₂ effects on AF vulnerability (AFV) and CANS function.

Methods

The protocol was approved by the Institutional Animal Care and Use Committee and conformed to Guide for the Care and Use of Laboratory Animals. We studied 10 Mongrel canines (20–30kg).

First surgery - radiotelemetry implantation and electrophysiology study:

Figure 1A diagrams the experimental timeline. Animals were induced with intravenous sodium methohexital, and anesthetized with 1–2% isoflurane for a left thoracotomy. A radiotelemetry device (Data Sciences International, Minneapolis, MN) was implanted to record HR and autonomic nerve activity (NA) from the left stellate ganglion (SNA), left cardiac vagus nerve (VNA), and GP (GPNA). Electrophysiology study (EPS) was performed to determine pacing thresholds, AERP, and AFV at three LA sites: anterior LA (ALA), LA appendage (LAA), and posterior LA (PLA) (Figure 1B) using an electrical stimulator.² GP were identified by: (1) visualization of epicardial fat pad at the antrum of the pulmonary vein (PV)⁴ (yellow arrow, Figure 1C) and (2) high frequency subthreshold stimulation (HFS) at 20Hz for 10 seconds,² that evoked a 5% decrease in HR. The chest was closed and the animal recovered for 24 hours prior to the ambulatory recordings of NA and HR.

Atrial fibrillation vulnerability score:

Figure 2A is an example of AF induced by atrial double extra-stimuli. All induced atrial arrhythmias with CL <200ms were defined as AF. AFV was quantitatively scored using an algorithm modified from the concept of AF *window of vulnerability*,² as further described in the data supplement.

Autonomic Nerve Activity (NA):

Ambulatory NA recordings began 24 hours after surgery to minimize residual NA suppression from anesthesia. Figure 2B is a sample of NA showing the association between ambulatory NA and HR. A 72-hour period of recordings were obtained at rest after the first surgery (Pre-LGP tx) and repeated after second surgery (Post-LGP tx) i.e., after LGP treatment with nCaCl₂ or vehicle control (sham). NA and HR were also recorded during exercise tolerance testing (ETT). Methods for analyzing NA¹ are detailed in the data supplement.

Echocardiography and Exercise Tolerance Testing:

Refer to the data supplement for details.

Second surgery - nCaCl₂ left-sided GP treatment and repeat electrophysiology study:

A second left thoracotomy was performed 3wks after first surgery (Figure 1A). EPS and HFS of the LGP (Figure 1B) were repeated (Pre-LGP tx). The LGP were each injected (Figure 1C and 1D) with either: 1) 1ml of nCaCl₂ consisting of PLGA particles (230 nm), carrying magnetite particles (14 nm) for histological tracing and a payload of 0.5mM CaCl₂ with trace L-glutamate, or 2) 1ml of vehicle control containing PLGA particles and magnetite only. The formulation was designed to produce local extracellular Ca⁺⁺

concentrations of 0.27mM at targeted GP sites.⁷ Injection produced a bleb in the fat pad without bleeding (Figure 1D), confirming an epicardial injection plane. After 1hr, the EPS was repeated (1 hr Post-LGP tx). Successful GP injections were confirmed by the loss of evoked HR response to HFS. The thoracotomy was closed and the animal recovered.

Final surgery - right-sided GP treatment and repeat electrophysiology study:

A left thoracotomy was performed 1 week after second surgery. EPS was repeated (1wk Post-LGP tx) to determine the 1 week effects of LGP treatment. A right thoracotomy was then performed to administer nCaCl₂ or the sham into the right PV GP (RGP). After 1 hour, EPS was repeated (1 hr Post-RGP tx). The animals were euthanized and tissues fixed in 4% formalin overnight, then stored in 70% alcohol.

Histological studies:

Tissue segments containing GP were paraffin embedded, sectioned, and stained with hematoxylin and eosin. Magnetite (Fe₃O₄) was added to nCaCl₂ to disclose localization of PLGA particles within GP (Prussian blue reaction). TUNEL staining was performed to confirm apoptosis. Immunostaining with Tyrosine Hydroxylase and Choline Acetyltransferase antibodies was used to differentiate adreno-cholinergic neurons,⁵ as further described in the data supplement.

Statistical analysis:

Statistical methods are described in detail in the data supplement. All data were expressed as mean $\pm$ SEM. A p-value of <0.05 was considered statistically significant.

Results

Subthreshold High frequency stimulation of GP:

Figure 3A illustrates transient HR slowing and induction of premature atrial complexes during HFS of GP. Figure 3B illustrates in the same animal, induction of AF during HFS of GP. Note that AF induction during GP HFS was not systemically quantified. HFS of GP decreased HR by 6 $\pm$ 2bpm during first surgery (Figure 3C, baseline), and 5 $\pm$ 2bpm during second surgery (Pre-LGP tx). Within 1 minute of nCaCl₂ injection, HR increased (111 $\pm$ 5 to 121 $\pm$ 6bpm, p<0.01) in all GP. nCaCl₂-treatment subsequently abolished the HR response to HFS of GP at 1 hour Post-LGP tx, 1 week Post-LGP tx, and 1 hour Post-RGP tx (p<0.05). In contrast, sham animals did not have a significant increase in HR at the time of GP injection (0.1 $\pm$ 1.4bpm) as compared with nCaCl₂ injection (p=0.03). HFS of sham-injected GP showed a transient loss of HR response at 1 hour (1.4 $\pm$ 1.4bpm vs pre-injection -7 $\pm$ 2.3bpm, p=0.03) but this was restored at 1 week (-4.3 $\pm$ 2.4bpm, p=0.1 vs pre-injection).

Effect of nCaCl₂-treatment on atrial effective refractory period:

Figure 4A is a summary of mean AERP changes after treatment with nCaCl₂ or vehicle control (sham). Mean AERP reflects average AERP of 3 LA regions. Supplemental Figure 1 details AERP in three LA regions. Table 1 summarizes all results. There was a significant decrease in mean AERP (p<0.0001) from first surgery (Baseline) to second surgery (Pre-

LGP tx). This decrease was observed at all LA sites (Table 1, Supplemental Fig. 1). After nCaCl₂-LGP treatment, mean AERP increased significantly at 1 week ($p=0.0004$). The increase in AERP was progressive, being significant in the PLA at 1 hour (1 hr Post-LGP tx), in the PLA and LAA at 1 week (1 wk Post-LGP tx) and in all sites at 1 hour after RGP treatment (1 hr Post-RGP tx). Furthermore, mean AERP at 1 hour after RGP treatment was greater than at baseline ($p=0.015$). In the sham group, AERP decreased in all sites between baseline and second surgery (Pre-LGP tx) ($p=0.001$). However, after sham treatment, mean AERP decreased further at 1 week Post-LGP tx ($p=0.04$ vs Pre-LGP tx) and remained decreased at all timepoints compared with baseline.

Suppression of POAF vulnerability:

Figure 4B shows vulnerability to AF (mean AFV) before and after treatment with nCaCl₂ or sham, reflecting average AFV of three LA regions. Supplemental Figure 2 details AFV changes per LA region. Table 2 summarizes all AFV results. Mean AFV increased from first surgery (baseline) to second surgery (Pre-LGP tx) ($p=0.008$). This increase was apparent at all LA sites (Table 2, Supplemental Figure 2). After nCaCl₂-LGP treatment, mean AFV decreased by 1 week post-LGP tx ($p=0.04$). However, this decrease was significant in the PLA ($p=0.02$) and LAA ($p=0.03$) but not in the ALA ($p=0.07$). Subsequent RGP-treatment further reduced mean AFV ($p=0.02$) and again, this was significant in the PLA (to 0, $p=0.01$) and LAA (to 0, $p=0.02$) but not in the ALA ($p=0.06$). In the sham group, mean AFV increased at second surgery (Pre-LGP tx) vs Baseline ($p=0.003$), and remained increased at all subsequent time-points ($p=0.004$, $p=0.002$, and $p=0.02$ respectively).

Evidence of autonomic modulation:

Figure 5 summarizes the effects of LGP treatment with nCaCl₂ vs sham on NA and HR during 24-hr of rest (5A and B) and 9-min of ETT (5C and D). The data obtained at two timepoints after 1st surgery (Pre-LGP tx) and after 2nd surgery (Post-LGP tx), represent NA in the ambulatory postoperative state without LGP treatment (after first surgery) and with LGP treatment (after second surgery). After nCaCl₂-LGP treatment (Post-LGP tx), resting SNA increased ($p<0.02$) whereas resting VNA ($p=0.18$) or GPNA ($p=0.16$) remained unchanged. Despite increased resting SNA (Fig. 5A), resting HR decreased ($p<0.01$) (Fig. 5B, Supplementary Fig. 3A), with loss of HR-SNA correlation ($R=0.09$). Heart rate variability (SDNN) at rest was unchanged (0.28 ± 0.037 to 0.32 ± 0.029 , $p=0.19$). During ETT (Fig. 5C), VNA ($p<0.05$) and GPNA ($p<0.02$) responses to exercise decreased whereas SNA response was not significantly different ($p=0.09$). However, HR response during exercise (Fig. 5D, Supplementary Fig. 3B) decreased ($p<0.01$) and was only modestly correlated with exercise SNA ($R=0.62$).

Sham treatment (Fig. 5A) resulted in no significant changes to resting SNA ($P=0.40$), resting VNA ($P=0.25$) and GPNA ($P=0.43$). Although resting HR (Fig. 5B) decreased ($p<0.03$), a change similar to after nCaCl₂ treatment, SDNN increased (0.26 ± 0.04 to 0.39 ± 0.06 , $p<0.006$) and resting HR remained tightly correlated with SNA ($R=0.99$), in contrast to the nCaCl₂ group. During ETT (Fig. 5C and D), SNA ($P=0.055$), VNA ($P=0.082$), GPNA ($P=0.13$) and HR response ($P=0.25$) were not significantly different after sham treatment. However, exercise HR and SNA remained correlated ($R=0.95$).

Effects of nCaCl₂ on Cardiac Function:

There were no acute changes in LVEF after nCaCl₂ treatment (58±3% vs 61±2% p=0.21).

Histology:

Prussian blue reaction revealed aggregated magnetite-containing nanoparticles localized to the epicardial fat layer containing GP (black arrows, Figure 6A) but not in the deeper atrial myocardium. All nCaCl₂-treated GP had evidence of neural somas (6B) exhibiting condensed cytoplasm and pyknotic nuclei (red arrow), consistent with apoptosis⁸ and confirmed by the positive TUNEL staining (red arrowheads, 6D). Sham-treated GP soma (6C) did not demonstrate cellular apoptosis. Figure 6E and F are stained with Choline Acetyltransferase (ChAT) (6E) and Tyrosine Hydroxylase (TH) (6F) respectively, demonstrating negative immunoreactivity in all nCaCl₂-treated GP soma. In contrast, 93±7% of somas in sham-treated GP soma were immunoreactive to ChAT (6G) (p=0.006). Quantitative analyses of immunoreactivity of neural bundle are described in the data supplement and supplementary figure 4.

Discussion

Epicardial injection of a nanoformulation (nCaCl₂) comprised of PLGA polymer encapsulant, carrying a payload of 0.5mM CaCl₂ with trace L-glutamate, into LA ganglionated plexi (GP), reversed the postoperative reduction in AERP and suppressed AF vulnerability in a canine model of POAF. The effects were evident by 1 hr due to acute GP suppression by PLGA burst release calcium and greater at 1wk, consistent with GP neuroapoptosis, but absent in vehicle control (sham) animals. Treatment with nCaCl₂ suppressed GP function and moderated heart rate response to increased SNA, consistent with autonomic modulation. The atrial electrophysiologic effect of nCaCl₂ is most apparent closest to targeted GP and cumulative with additional treatment of RGP to LGP. There was no nanoparticle infiltration into atrial myocardium. We conclude that targeted GP suppression by nCaCl₂ suppresses POAF by reversing postoperative AERP reduction and protects the heart against postoperative sympathetic surge.

Neuronal excitotoxicity:

The nCaCl₂-induced GP excitotoxicity stems from its ability to increase extracellular calcium concentration, increase the transmembrane calcium gradient, promoting calcium internalization and neuroapoptosis.⁶ L-glutamate facilitates calcium internalization by activating neuronal NMDA receptors.⁶ Increased intracellular calcium activates an enzymatic (e.g. calpain) cascade which eventually causes neuroapoptosis.⁶ This localized ganglion cell death, could be characterized as a targeted chemical ablation of GP. Histological data showing preferential effect of nCaCl₂ on neural somas versus axonal bundles (data supplement, supplementary figure 4) underscore the concept of targeted therapy.

Advantages and safety of targeted nanomedicine therapy:

Encapsulation of CaCl₂ within nanoparticles has several therapeutic advantages in targeted GP modulation.⁹ First, PLGA has a burst release within minutes followed by sustained

release of payload for days to weeks,^{13,18} prolonging GP exposure to CaCl_2 . *In vitro* studies⁷ demonstrated that 30% of the PLGA payload is released within 30 minutes, and another 40% by 20 days. The increased HR observed within minutes of nCaCl_2 is consistent with the acute excitatory phase of calcium-glutamate excitotoxicity. Second, nanoparticles concentrate and stabilize the payload at the target site by virtue of their small diameter (230 ± 101 nm) creating a large surface area to volume ratio which facilitates sufficient neuronal calcium internalization.

Autonomic modulation and POAF suppression:

We made several key observations. First, nCaCl_2 -LGP treatment is sufficient to reverse the postoperative reduction in AERP and increase in AFV following cardiac surgery. Second, the anti-arrhythmic action of nCaCl_2 is evident within 1 hour closest to the GP (in the PLA), becoming more widespread and pronounced at 1 week, consistent with the time course of an apoptotic process.⁶ Third, sham control animals had transient loss of HR response to GP HFS, and transient (non-significant) decrease in AFV at 1 hour (Fig. 4B), possibly due to mechanical perturbation of GP by the 1ml aqueous injectate. Fourth, nCaCl_2 -LGP treatment were magnified by nCaCl_2 -RGP treatment indicating greater AF suppression when more GP were targeted, similar to prior RF studies^{10,11} favoring a more extensive anatomic approach to GP ablation. Fifth, nCaCl_2 -GP treatment modulates the autonomic balance within the CANS, evidenced by: (a) reversed AERP remodeling, (b) moderation of HR response to increased SNA, and (c) reduced exercise GPNA after nCaCl_2 . Following LGP treatment, HR was reduced in nCaCl_2 -treated animals despite increased SNA. On the other hand, HR was also reduced in sham-control animals, but was concordant with SNA. While nCaCl_2 -LGP treatment resulted in some degree of sympathetic blockade, circadian HR variability and HR response to exercise remain preserved (Supplemental Figure 3), indicating cardiac autonomic modulation not denervation. These autonomic changes were nevertheless significantly different from the increased heart rate and reduced heart rate variability reported after RF ablation.^{10,11} The discrepancy reflects two different modes of GP modulation (calcium vs RF), treatment of the left subset of LA GP versus all LA GP or RA GP in other studies^{11,10} or comparisons with different denominators (i.e. between two post-thoracotomy states vs between pre- and post-RF ablation states).^{10,11} Because nCaCl_2 -induced neuroapoptosis is a phased process beginning with cellular excitation and culminating in cell death,^{6,8} we hypothesize that increased SNA is a result of activation of cardio-cardiac reflexes¹² elicited by excitotoxicity of GP soma. In contrast, RF energy unselectively destroys all neural and myocardial tissue. Any reflex changes triggered might conceivably be attenuated because of more extensive destruction of the components of the reflex limb.

Preferential effect of nCaCl_2 on GP soma.

Immunostaining demonstrated complete loss of immunoreactivity in GP somas but only partial loss of immunoreactivity in neural bundles (data supplement, supplementary figure 4). These data indicate a preferential effect of nCaCl_2 on local soma rather than trafficking neurons, underscoring the specificity of GP modulation compared with RF ablation. A potential mechanism for this specificity is the increased role of extra-synaptic (versus synaptic) NMDA receptors in neuro-excitotoxicity,¹³ and their preferential localization in

neural soma and non-synaptic dendrites versus axons.¹³ The targeted nature of nCaCl₂ provides a further explanation for the different autonomic reflexes elicited after nCaCl₂ vs RF treatment of GP.

Comparisons with Botulinum Toxin

Botulinum toxin (botox) is a cholinergic neuron blocker that binds to presynaptic cholinergic neurons and prevents acetylcholine release for 3–6 months. Yet, botox injection into ganglia suppresses AF following CABG well beyond that period.^{14,15} The mechanism(s) are unclear. Data from a canine model of pacing-induced AF suggests that even transient cholinergic neuron suppression may cause a reset of the CANS¹⁶ sufficient to render longer term protection against AF. Restani et al¹⁷ discovered extensive retrograde axonal transport of botox from the neuromuscular junction of primary motor neuron cultures. We speculate that retrograde axonal transport of botox from GP to the CANS network could explain the more sustained resetting of the CANS. In contrast, due to evident neuroapoptosis in our study and the limited regenerative capacity of the CANS, we suspect that nCaCl₂ can confer persistent neuromodulation and AF suppression. These unresolved questions merit further investigation.

Limitations

We were unable to treat all 4 LA GP simultaneously due to the limitations of a unilateral left thoracotomy approach necessitated by survival surgeries. Nevertheless, our sequential approach tested the relative contribution of LGP then RGP to AF vulnerability. We chose a 1 week duration post-treatment coinciding with the time course of apoptosis⁶ and peak incidence of POAF.¹⁸ However, a chronic study would be needed to substantiate the presence of chronic neurosuppression and reveal any long term side effects. Our survival surgeries necessitated the use of isoflurane anesthesia, which suppresses sympathetic nerve activity in a dose-dependent manner,¹⁹ and could have diminished GP responses to HFS.² Non-invasive manual BP readings did not demonstrate any off-target effects of nCaCl₂ on BP. Finally, multi-site GP recordings might be more informative on the state of the intrinsic CANS function.

Conclusion

Epicardial Injection of nanoformulated calcium chloride into LA ganglionated plexi, suppresses GP function and POAF. The mechanism is burst-then-slow release of calcium from nanoparticles that results in calcium-induced acute neurotoxicity and neuroapoptosis. This targeted GP treatment exerts an anticholinergic modulation on the atrial electrophysiology (increasing AERP) and provides cardioprotection against the postoperative sympathetic surge, sufficient to reverse the atrial electrophysiologic remodeling triggered by cardiac surgery and suppress AF. This treatment is easily administered without observed side effects, atrial damage or effects on ventricular function. We conclude that nanoformulated calcium administered during open heart surgery is a promising therapeutic option to prevent POAF.

Clinical Translation

POAF is one of the most prevalent complications of cardiac surgery responsible for prolonged hospital stays and additional costs of US\$2 billion in the US alone.²⁰ Although POAF peaks during the first postoperative week, it is an independent predictor of late AF.²¹ Nanoformulated calcium specifically targets the intrinsic CANS, a major pathophysiologic driver for POAF. Delivered by direct epicardial injection into GP, the nanoparticles release their calcium in a burst and timed-release manner, increases local extracellular calcium concentration in the GP and causes neuroapoptosis. This targeted therapy modulates intrinsic CANS function by reversing atrial electrophysiologic remodeling caused by postoperative injury and repair, moderates cardiac response to sympathetic surge and suppresses POAF. Long term studies will be needed to substantiate the durability of its anti-arrhythmic action.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We acknowledge Martha Joslyn MS, Li Wang PhD, Anindita Das PhD, Robert Lippman, MD, Austin Minuto, and Andrew Worley for their assistance.

Funding Sources

This study is supported by Virginia Commonwealth Commercialization Fund (CRCF: MF16-041-LS), American Heart Association (16SDG3128001), and National Institutes of Health (1R56HL133182-01).

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Figure 1. Experimental Protocol.

(A) Timeline of study. (B) Posterolateral view of LA structures. LAA and ALA (not visible) are located anterolateral to LGP. Abbreviations: LSPV and LIPV: left superior and inferior pulmonary veins; SLGP and ILGP: superior and inferior left GP; LOM: Ligament of Marshall; PLA, ALA, LAA: posterior LA, anterior LA, LA appendage, respectively. GP (yellow arrow) prior to (C) and after injection of nCaCl₂ (D), showing the characteristic bleb.

Figure 2.

(A) Programmed stimulation to induce AF. (B) Representative NA recordings from left stellate ganglion (SNA), cardiac vagal nerve (VNA), and left inferior ganglionic plexi (GPNA) during normal activity.

Figure 3. High Frequency Subthreshold Stimulation (HFS) of GP.

(A) Example of HFS resulting in transient HR slowing from 133 to 122 bpm, and triggering PACs. (B) In the same animal, HFS of GP also triggered PAF. (C) Heart rate changes are abolished after treatment of GP with nCaCl₂.

Figure 4: Effects of nCaCl₂ on Atrial Effective Refractory Period (AERP) and Atrial Fibrillation Vulnerability (AFV).

Sequential changes in AERP and AFV with nCaCl₂ or vehicle control (sham) treatment, from first surgery (**baseline**), to second surgery before (**Pre-LGP tx**) and 1 hr after LGP treatment (**1hr Post-LGP tx**), to final surgery (**1wk Post-LGP tx**) including the treatment of RGP (**1hr Post-RGP tx**). Mean AERP and AFV values represented the respective average of 3 LA regions.

Figure 5: Effects of Left GP Treatment with nCaCl₂ on Autonomic Nerve Activity and Heart Rate.

Average NA (**A**) and HR (**B**) during 24-hours of rest and during 9 minutes of exercise treadmill testing (ETT) (**C and D**) before (Pre-LGP tx) and after (Post-LGP tx) nCaCl₂ treatment to left GP. The nCaCl₂ group was compared with vehicle control (sham) group. * P<0.05 ** P<0.01 vs Pre-LGP tx.

Figure 6. Histological Analyses

(A): Prussian Blue reaction of LGP demonstrating blue coloration of aggregated magnetite-containing nanoparticles (black arrow). (B) Cellular apoptosis characterized by pyknotic nuclei with condensed cytoplasm. (C) H+E stain of GP in sham group showing normal soma in GP. (D): TUNEL staining (red arrows) within nuclei within apoptotic neuron. Negative immunoreactivity of somas to Choline Acetyl Transferase (E) and Tyrosine Hydroxylase (F) 1-week after $n\text{CaCl}_2$ -treatment, compared ChAT-positive somas in sham-treated GP (G). Further immunostaining analyses in data supplement.

Table 1.
Changes in Atrial effective refractory period (AERP) after nCaCl₂ vs sham treatment.

Sequence from first surgery (baseline), to second surgery before (Pre-LGP tx) and 1 hr after LGP treatment (1 hr Post-LGP tx), to final surgery (1wk Post-LGP tx) including the treatment of RGP (1 hr Post-RGP tx).

Treatment	Area	Baseline	Pre-LGP tx vs. Baseline	1hr Post-LGP tx vs. Baseline vs. Pre-LGP tx	1wk Post-LGP tx vs. Baseline vs. Pre-LGP tx	1hr Post-RGP tx vs. baseline vs. Pre-LGP tx
nCaCl ₂	LAA	125±6ms	115±6ms <i>p=0.02</i>	118±9ms <i>p=0.28</i> <i>p=0.32</i>	130±3ms <i>p=0.32</i> <i>p=0.028</i>	135±9ms <i>p=0.11</i> <i>p=0.022</i>
	PLA	130±4ms	97±11 ms <i>p=0.009</i>	118±8ms <i>p=0.22</i> <i>p=0.026</i>	128±2ms, <i>p=0.77</i> <i>p=0.02</i>	140±4ms, <i>P=0.26</i> <i>P=0.003</i>
	ALA	132±8ms	102±11ms <i>p=0.04</i>	108±8ms <i>p=0.05</i> <i>p=0.7</i>	120±4ms <i>p=0.23</i> <i>p=0.12</i>	145±5ms <i>p=0.65</i> <i>p=0.012</i>
	Mean	129±3ms	104±5ms <i>p<0.0001</i>	115±5ms <i>P=0.02</i> <i>P=0.06</i>	126±2ms <i>P=0.48</i> <i>P=0.0004</i>	143±4ms <i>P=0.015</i> <i>P=0.002</i>
Sham	LAA	113±3ms	87±3ms <i>P=0.008</i>	80±6ms <i>P=0.007</i> <i>p=0.5</i>	77±3ms <i>p=0.004</i> <i>p=0.09</i>	87±6ms <i>P=0.03</i> <i>p=0.9</i>
	PLA	107±3ms	83±3ms <i>P=0.009</i>	96±3ms <i>p=0.12</i> <i>p=0.04</i>	77±3ms <i>p=0.01</i> <i>p=0.13</i>	80±1ms <i>P=0.03</i> <i>p=0.5</i>
	ALA	113±3ms	93±3ms <i>p=0.03</i>	80±6ms <i>P=0.03</i> <i>P=0.16</i>	83±3ms <i>P=0.03</i> <i>p=0.2</i>	77±7ms <i>P=0.01</i> <i>P=0.18</i>
	Mean	111±4ms	88±3ms <i>p=0.001</i>	86±6ms <i>P=0.04</i> <i>p=0.58</i>	82±2ms <i>P=0.01</i> <i>p=0.04</i>	81±3ms <i>P=0.01</i> <i>p=0.07</i>

Table 2.Changes in Atrial Fibrillation Vulnerability Scores (AFV) after nCaCl₂ vs sham treatment.

Treatment	Area	Baseline	Pre-LGP tx vs. Baseline	1hr Post-LGP tx vs. Baseline vs. Pre-LGP tx	1wk Post-LGP tx vs. Baseline vs. Pre-LGP tx	1hr Post RGP tx vs. Baseline vs. Pre-LGP tx
nCaCl ₂	LAA	1.7±1.3	8.7±3.4 <i>p</i> =0.03	0 <i>p</i> =0.42 <i>p</i> =0.04	1.6±1 <i>p</i> =0.77 <i>p</i> =0.03	0 <i>p</i> =0.2 <i>p</i> =0.02
	PLA	0.8±0.5	9±2.9 <i>p</i> =0.01	3±2.6 <i>p</i> =0.42 <i>p</i> =0.032	2.2±1.4 <i>P</i> =0.37 <i>P</i> =0.02	0 <i>p</i> =0.19 <i>p</i> =0.01
	ALA	0.3±0.3	10.5±5.3 <i>p</i> =0.02	6±3.7 <i>p</i> =0.25 <i>p</i> =0.11	5.4±2.1 <i>p</i> =0.16 <i>p</i> =0.07	4.8±3.2 <i>p</i> =0.08 <i>p</i> =0.06
	Mean	0.9±0.5	9.4±2.8 <i>p</i> =0.008	3±2.1 <i>p</i> =0.15 <i>p</i> =0.08	3.1±0.9 <i>P</i> =0.16 <i>P</i> =0.04	2.4±1.6 <i>P</i> =0.55 <i>P</i> =0.02
Sham	LAA	5±5	14.7±2 <i>p</i> =0.05	12±5 <i>p</i> =0.09 <i>p</i> =0.8	21.3±10.3 <i>p</i> =0.07 <i>p</i> =0.62	17.3±8 <i>p</i> =0.11 <i>p</i> =0.54
	PLA	3.3±0.7	20.7±8.2 <i>P</i> =0.02	9±4.2 <i>p</i> =0.06 <i>p</i> =0.15	25.7±5 <i>P</i> =0.02 <i>p</i> =0.77	23±12.7 <i>p</i> =0.04 <i>p</i> =0.8
	ALA	1±1	10±4.4 <i>p</i> =0.007	13±3.8 <i>P</i> =0.02 <i>p</i> =0.45	26±3.5 <i>p</i> =0.004 <i>p</i> =0.03	24.3±9.8 <i>p</i> =0.04 <i>p</i> =0.1
	Mean	3.1±2.1	15.1±4.2 <i>p</i> =0.003	11.3±2 <i>P</i> =0.004 <i>p</i> =0.22	24.3±5.7 <i>P</i> =0.002 <i>P</i> =0.05	21.6±10.1 <i>p</i> =0.02 <i>p</i> =0.12