

Atrial Connexin (40 And 43) Remodeling in Atrial Fibrillation

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THESIS

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DEDICATION

To Vishnucharan Panneerselvam, for being my voice of reason, my friend, my foe at times, my counselor and for tolerating everything through the tough phases being miles away. You are truly my life raft.

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Acknowledgment, it is said would bring in recognition to the acknowledged. But these few lines written here are not with that purpose but, to express in a small way a sense of gratitude to all concerned.

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LIST OF ABBREVIATIONS

SA	SINO-ATRIAL
AV	ATRIOVENTRICULAR
ECG	ELECTROCARDIOGRAM
AF	ATRIAL FIBRILLATION
LAA	LEFT ATRIAL APPENDAGE
AP	ACTION POTENTIAL
CV	CONDUCTION VELOCITY
ERP	EFFECTIVE REFRACTORY PERIOD
WL	WAVELENGTHS
EAD	EARLY AFTER-DEPOLARIZATION
DAD	DELAYED AFTER DEPOLARIZATION
APD	ACTION POTENTIAL DURATION
Cx43	CONNEXIN 43
Cx40	CONNEXIN 40
Cx45	CONNEXIN 45
ROS	REACTIVE OXYGEN SPECIES
NOS	NITRIC OXIDE SYNTHASE
ZO-1	ZONA OCCLUDENS-1
ACE	ANGIOTENSIN CONVERTING ENZYME
MnSOD	MANGANESE SUPEROXIDE DISMUTASE
CHF	CONGESTIVE HEART FAILURE
PLA	POSTERIOR LEFT ATRIUM

LA LEFT ATRIUM
RA RIGHT ATRIUM
PV PULMONARY VEIN
HRP HORSE RADISH PEROXIDASE
SDS SODIUM DODECYL SULPHATE
CTCF CORRECTED TOTAL CELL FLUOROSCENCE
RAA RIGHT ATRIAL APPENDAGE
ATP ATRIAL TACHYPACING

SUMMARY

Background: Atrial Fibrillation (AF) is an irregular, rapid, and disorganized electrical and contractile activity of the atria that affects approximately 2.5 million Americans and is the most common, sustained arrhythmia. AF has been shown to be an independent risk factor for thromboembolism. Myocardial electrical continuity is assured by gap junctions, intercellular connections that provide low resistance pathway via specialized hemichannel subunit proteins called connexins. Connexin40 and connexin43 are the principal atrial gap junctional subunits. Reduction in gap junctional proteins (connexin43 and 40) may play an important role in reducing myocyte coupling, resulting in a reduction in conduction velocity of propagating action potential and providing a substrate for AF. In addition, gap junctional impairment may be linked to the reduced contractility of the left atrium in AF. It is known that the posterior left atrium (PLA) is an important source of AF and that the majority of thromboembolic events originate from left atrial appendage (LAA) as LAA contractility is more compromised than other parts of the LA. I sought to determine the changes in the protein levels of Cx43 and Cx40 in the PLA and LAA tissues in a canine congestive heart failure (CHF) model associated with AF. Previous studies have reported that AF is associated with increased levels of Reactive Oxygen Species (ROS) and that mitochondrial oxidative stress may decrease connexin levels via oxidative activation of a tyrosine kinase, c-Src. Therefore, mitochondrial levels of ROS were also measured the PLA and LAA regions of the dog heart.

Methods: Control (n=4) and tachypacing induced CHF dogs (n=7) with increased propensity to AF were studied. Western blot analysis of Cx43 and Cx40 protein levels at PLA and LAA

SUMMARY (Contd...)

tissues of both control and CHF dogs were performed. MitoSOX red was used to stain isolated myocytes for detection of mitochondrial superoxide. Stained isolated myocytes were studied under confocal microscope, and analyses were performed using Image J software. A Student t-test was performed to record statistically significant values. $P < 0.05$ values were considered to be statistically significant.

Results: A 43.9% decrease in Cx43 protein and a 3.5% decrease in Cx40 protein in PLA tissue of CHF dogs compared to control (P =not significant) were observed. A more prominent decrease in Cx43 (88.4%) and Cx40 (53.1%) were detected in LAA tissue of CHF dogs compared to control ($P < 0.001$ and $P < 0.01$ values respectively). AF was also associated with significant increase in mitochondrial ROS levels in both LAA (3.2 ± 0.8) and PLA (1.9 ± 0.3) of CHF dogs, when compared to their controls (0.7 ± 0.1 , LAA and 0.4 ± 0.03 , PLA, $P < 0.01$). ROS levels were higher in CHF LAA than in CHF PLA by 43%.

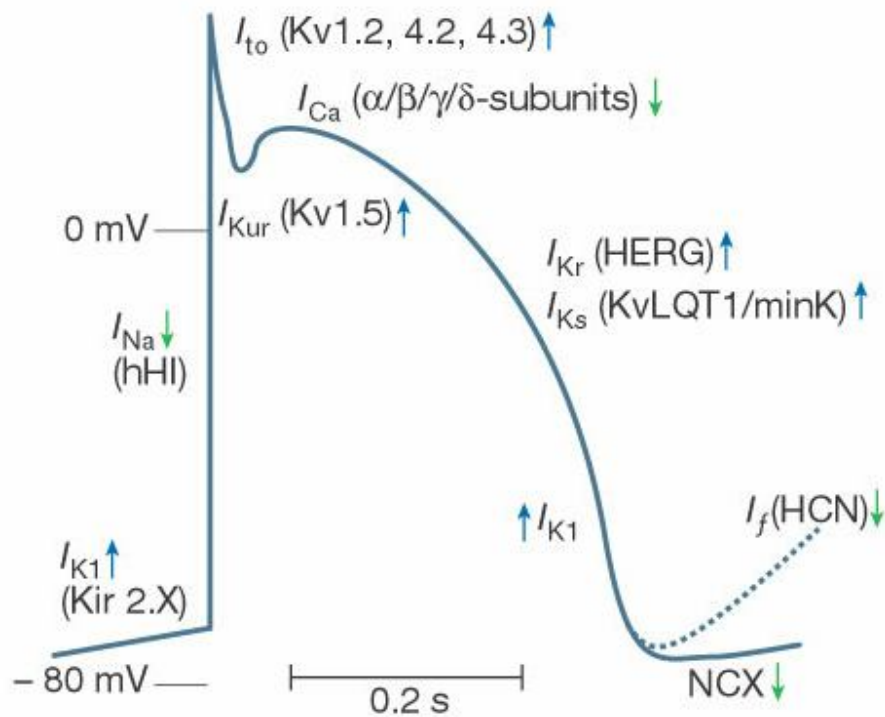
Conclusion: AF is associated with increase in mitochondrial ROS and decrease in both Cx43 and Cx40. These abnormalities were all more prominent in LAA than in PLA of CHF dogs. Mitochondrial oxidative stress and gap junctional remodeling may be important substrate for AF and the associated abnormal contractility. The regional heterogeneity in those abnormalities may provide further insight into the mechanisms of thromboembolism in AF

1) INTRODUCTION

1.1) NORMAL ELECTROPHYSIOLOGY OF THE HEART:

In normal heart, the pacemaker called the sino-atrial (SA) node, present in the right atrium, generates action potentials that propagate from the right atrium to the left atrium via the Bachmann's bundle causing the atria to contract. The action potential travels then through the atrioventricular (AV) node with some delays and to the bundle of His and the Purkinje fibers, causing the ventricles to contract simultaneously. Normal and rapid propagation of action potential is required for effective and simultaneous contractions of atria and then ventricles.

The resting membrane potential of a normal heart muscle cell is around -80 mV. Depolarization occurs when the sodium channels open rapidly, which causes an influx of sodium ions and depolarization the membrane potential (Figure 1.1). This is followed by the quick inactivation of the sodium ion channels and opening of the potassium ion channels causing the membrane potential to repolarize. This is the initial hyperpolarizing phase. At this point, calcium channels are also activated, which causes calcium-induced-calcium release initiating excitation-contraction coupling. This contributes the plateau phase of the action potential. Subsequently, delayed rectifier K^+ channels are activated, and the calcium channels get deactivated causing the termination of the plateau phase. The final repolarization phase is mediated by the outward rectifying potassium channel that brings back the membrane potential to its resting membrane potential.



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Figure 1.1) Action potential of an atrial myocyte showing the principal currents that flow in each phase, with the corresponding subunit clones shown in parentheses. Downward arrows represent inward ion or current movement, upward arrows represent outward ion or current movement(1).

Activation of cardiac myocytes and propagation of the action potential in the above mentioned manner creates a pattern of electrical activity at the heart, which can be recorded on the surface of the body with a 12-lead electrocardiogram (ECG). Normal activation of the heart usually creates a P wave that represents activation of atria, a QRS complex that represents activation of ventricles, and then a T wave that represents repolarization of ventricular myocytes.

Repolarization of atrial myocytes does not have an identifiable wave on the surface ECG because it falls within the QRS complex.

1.2) ATRIAL FIBRILLATION:

1.2.1) Atrial Fibrillation:

Atrial fibrillation is defined as rapid, disorganized and irregular activity of atria. During AF, the atrial rate rises to 350-600 beats per minute (bpm) from 60-100 bpm. AF is characterized by the absence of regular P waves or rapid fibrillatory P waves on an ECG. The ventricular response is variable and the ventricular rate depends on the AV conduction. Because of the asynchrony of the atria resulting from the improper initiation and propagation of action potential, atrial contractility is impaired and abnormal.

1.2.2) Epidemiology of Atrial Fibrillation:

Atrial fibrillation (AF) is the most commonly sustained arrhythmia that is associated with increased morbidity and mortality(2-4). AF incidence has been found to increase with age from less than 0.5 per 1000 person-years in patients younger than 50 years to approximately 10 per 1000 person years in patients older than 70 years(5). The Framingham heart study showed that the lifetime risk for the development of AF was 1 in 4 for men and women 40 years or older. Also, AF is found to be more common in men(6).

1.2.3) Types of Atrial fibrillation seen in clinical practice:

Atrial fibrillation can be broadly classified into two main types, first onset and chronic form (7). The chronic form is further subdivided into 3 types namely,

Paroxysmal: In this type of AF, the episodes are self terminating, and they usually last for less than 48 hours .

Persistent: Until some effort is taken to terminate the episode, it remains persistent.

Permanent: It refers to an irreversible state where reverting to normal sinus rhythm is not possible.

1.3) THROMBOEMBOLISM IN ATRIAL FIBRILLATION:

Atrial fibrillation is associated with increased risk of thromboembolism and stroke(8). The risk of stroke after being diagnosed with AF increases from 1.3% to 5.1% per person-year as age increases from 50 to 90 years(9). Virchow's triad (endothelial dysfunction, blood stasis or abnormal flow, and hypercoagulability) provides the basis for thrombosis in AF(10). Impaired contractility of the left atrium and in particular left atrial appendage (LAA) may cause blood stasis, and therefore, it may contribute as a major factor for increased thromboembolism risk in AF(11-14). Reduced blood flow velocities in the LAA have been linked to the formation of thrombi in AF patients(15). Patients are treated with systemic anticoagulants or sometimes the left atrial appendage is either clamped or ablated in order to prevent the formation of blood clots(16;17).

1.4) BASIC MECHANISMS OF ATRIAL FIBRILLATION:

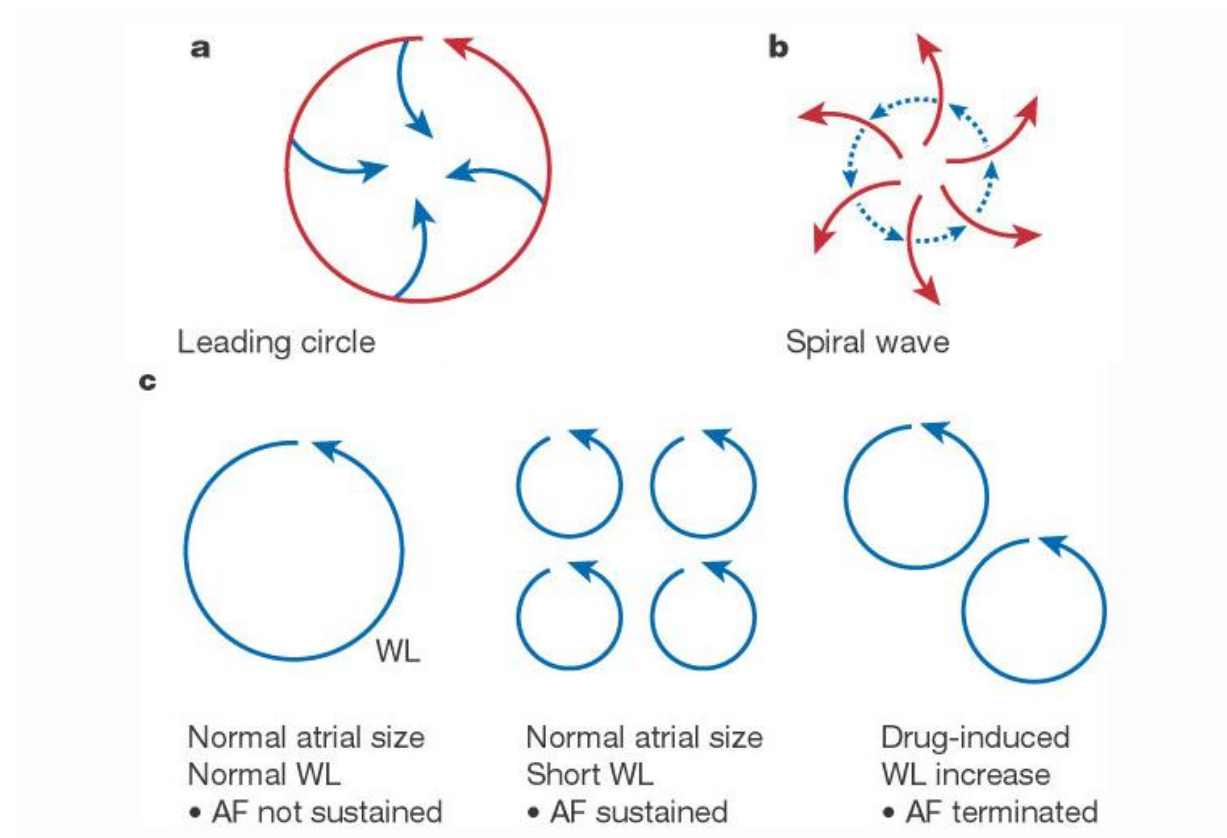
The mechanisms of AF can be divided to those that cause rapidly firing ectopic foci and those that cause wave break and reentry. The reentry mechanism of AF may appear as multiple

circuits, a single spiral wave, wave breaks or other forms in all of which the underlying abnormality is a unidirectional block or inhomogeneous reduction in conduction velocity.

1.4.1): Reentry

Reentrant mechanism plays a major role in maintaining AF. Reentry occurs when the following conditions are met: 1) A unidirectional block that is present in the impulse conducting pathway; 2) Slow conduction in the region surrounding the block; 3) Delayed activation of tissue beyond the block; and 4) Re-excitation of the tissue next to the block. Consider a case of an action potential (AP) propagating through two branches of which one of the branches contains a unidirectional block that is capable of conducting impulses in the retrograde direction but not anterograde while the other branch has slow conduction. As the action potential enters both the branches, the branch containing the block fails to conduct. The AP entering the slow conducting branch passes through and re-excites the branch containing the unidirectional block since it conducts the AP in retrograde fashion. As a result, a reentrant circuit is established that is self sustaining and can depolarize other parts of the heart causing tachyarrhythmia. The presence of excitable tissue in front of the head of the propagating wave helps in the continuation of the reentrant circuit(18). Reentry can be conceptualized as either a leading circle or a spiral wave. Activation of spiral waves (rotors) is organized around a core, which remains unstimulated because of pronounced curvature of the spiral(19). This curvature also reduces the conduction velocity and thereby creates a block(20). The leading circuit reentrant theory suggests that the functional reentrant circuits establish themselves in the minimum path length of reentry(18). The

minimum path length of reentry is determined by the product of the effective refractory period (ERP) and the conduction velocity (CV).



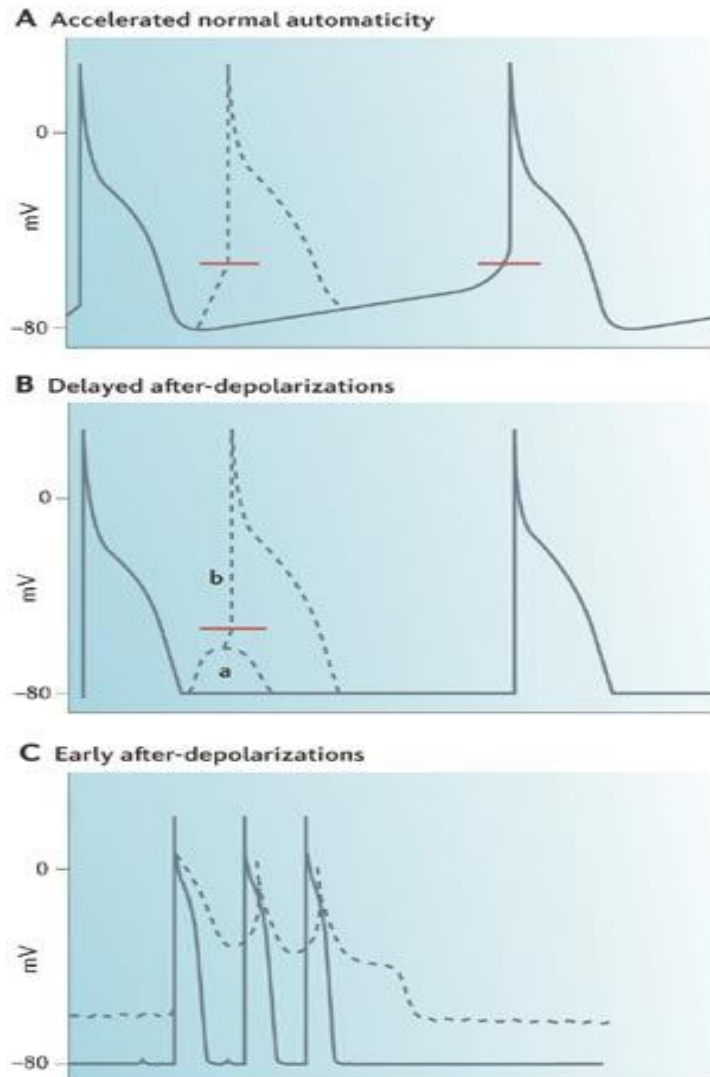
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a, Mechanism of functional re-entry in the leading circle model¹⁰. **b**, Spiral wave model of re-entry. **c**, Role of wavelength in the stability of atrial fibrillation according to the leading circle model. The size of functional re-entry circuits depends on the wavelength. Short wavelengths allow several simultaneous circuits to be maintained, favouring continuation of atrial fibrillation. Drugs that increase the wavelength reduce the number of circuits that can be accommodated, favouring termination of atrial fibrillation. CV, conduction velocity; PL, path length; RP, refractory period; WL, wavelength (1).

Effective refractory period is duration during which a new action potential cannot be initiated however large the stimulus may be. ERP protects the heart by preventing multiple action potentials from occurring at the same time. In other words it limits the frequency of depolarization and therefore the heart rate. Hence, from this relationship, it is evident that short ERPs and slow CVs gives rise to short circuits length therefore allowing multiple reentrant circuits to co-exist in a particular area. Also, large atrial size, which usually accompanies AF, allows for multiple reentrant circuits to co-exist.

1.4.2) Rapid Ectopic Activity:

Rapid ectopic activity is thought to initiate AF. Rapid focal activity can be caused by either triggered activity or enhance automaticity. In enhanced automaticity, the myocyte reaches depolarization threshold in phase 4 of its AP earlier than normal resulting in increased automatic rate of firing. Triggered activity arises from after-depolarizations that are basically low frequency (around 5 Hz) depolarizing oscillations in membrane voltage (21). They can be either early afterdepolarization (EADs) or delayed afterdepolarization (DADs). EADs occur only at low frequency stimulations while DADs occur only at high frequency stimulations. Manifestations of EADs occur because of reactivation of L-type calcium channels during repolarization secondary to the prolongation of AP as a result of reduced repolarization reserve (21). EADs occur during the repolarization phase of the action potential to be more precise, phase 2-3 of the action potential. DADs occur because of the spontaneous release of Ca^{2+} from an overloaded sarcoplasmic reticulum(22). The calcium ions then exit the cell via the $3\text{Na}^+/1\text{Ca}^{2+}$ exchanger which causes the influx of sodium ions thereby depolarizing the



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A) Abnormal automaticity is represented by the dashed line and normal automaticity is represented by the solid line **B)** After-depolarizations of a typical ventricular cell (DADs; dashed line, **a**) and are generally because of abnormal intracellular Ca^{2+} release that results in an excessive inward current carried by the Na^+ , Ca^{2+} exchanger. If the DAD is large enough to reach threshold, it will cause abnormal extra beating (dashed line, **b**) before the next expected action potential (solid line). **C)** When after-depolarizations occur before complete AP repolarization, they are called 'early after-depolarizations' (EADs). APs of two cells are shown: a cell (upper trace) that fails to repolarize, generating repeated EADs that cause the adjacent cell to fire repeatedly (lower trace). (23)

membrane and sparking a new action potential(22). DADs are seen after complete repolarization of the action potential.

1.5) TREATMENTS FOR ATRIAL FIBRILLATION:

There are two potential approaches to atrial fibrillation management—restoration and maintenance of sinus rhythm (rhythm control), and leaving patients in atrial fibrillation but controlling the ventricular response (rate control)(24). Traditional antiarrhythmic drugs are class I agents that inhibit sodium (Na^+) currents and class III agents that inhibit potassium (K^+) currents(24). Class I drugs suppress ectopic activity that is associated with EADs and DADs by reducing excitability, therefore preventing EADs to reach their threshold. They also suppress automaticity by blocking Na^+ channels. Class III drugs suppress reentry by increasing refractoriness but do not affect conduction velocity. Class I drugs promote reentrant mechanisms by slowing conduction velocity, and class III drugs promote EADs by lengthening the action potential duration (APD) and QT interval(24).

Atrial fibrillation is also treated surgically by tissue ablation. Atrial fibrillation ablation is a therapeutic technique that uses radiofrequency energy or freezing to destroy atrial tissue that is involved in the propagation of the arrhythmia(25). Radiofrequency ablation generates an alternating electrical current that passes through myocardial tissue, creating heat energy that conducts to deeper tissue layers. Cryoablation destroys tissue by freezing. The main principal of catheter ablation of tissue is electrically disconnecting the arrhythmic tissue from the tissue substrate. Usually the tissue ablated is the pulmonary vein which has been found to be the major driver of arrhythmia(25-27). Nevertheless, many complications arise from catheter ablation.

Cerebrovascular thromboembolism can occur because of char or clot formation caused by the catheter at the site of ablation(25). Cardiac perforation can occur because of improper catheter movement or excessive radiofrequency application.

Presently available therapeutic approaches have limited efficacy and considerable risks, so new strategies and therapeutic targets to prevent and manage the condition are urgently required(28).

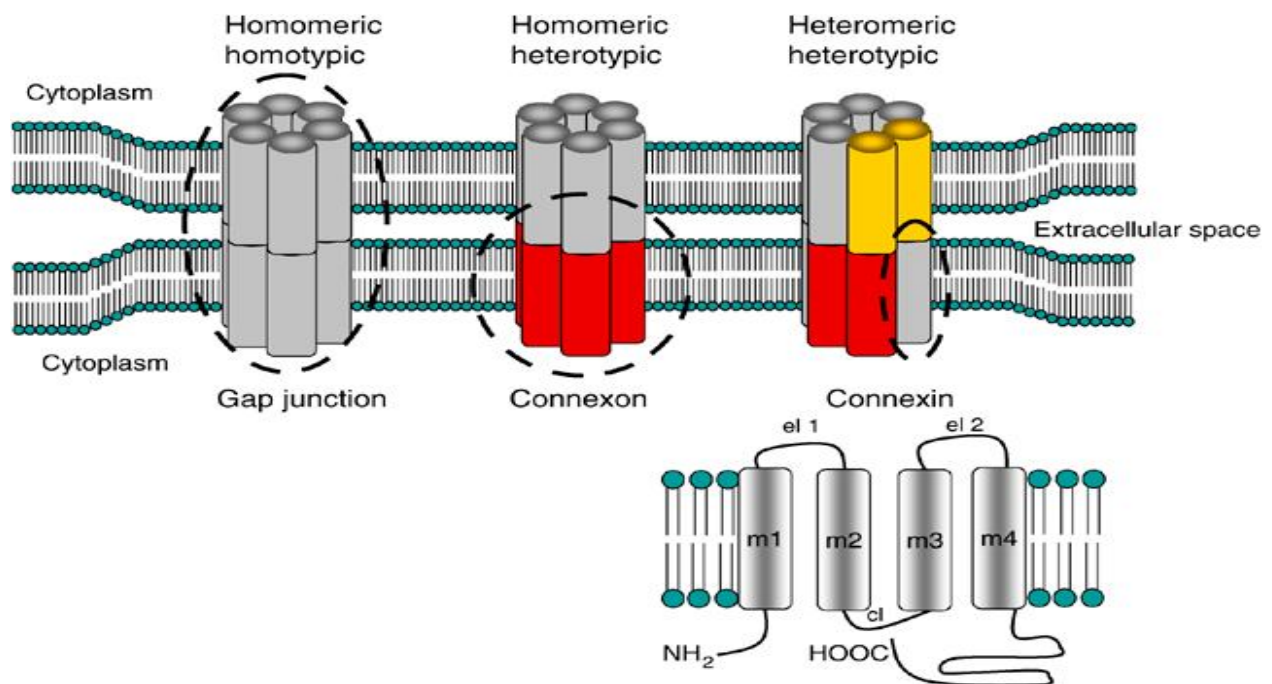
1.6) ROLE OF GAP JUNCTIONS IN REENTRY AND PROPAGATION OF FOCAL ACTIVITIES

1.6.1) Gap Junctions:

Gap Junctions are specialized structures that allow intercellular communication. Through gap junctions, cells can send and receive signals, and gap junctions help in cell growth, differentiation and electrical conduction. Gap junctions play a vital role in cardiac muscle. They provide a low resistance pathway for the propagation of action potential. Each gap junction is composed of two hexameric structures called the connexons. These connexons are made up of six transmembrane proteins, connexins. There are three main types of connexin protein found in the atrial gap junctions- connexin40 (Cx40), connexin43 (Cx43) and connexin45 (Cx45). Expression levels of connexin40 in the right atrium are 2 to 3 fold higher than the left.(29) In contrast, connexin43 is homogeneously distributed throughout the right and the left atria and has comparable levels to connexin40 in the right atrium(29). Because of high cross reactivity of connexin45 antibodies with other connexins, it has been difficult to quantify the connexin45 protein in either normal or pathological atrial tissue(29). Cx40 gap junctions have relatively high

unitary conductance (200pS) (30) and they are mildly voltage and pH dependent(31). On the other hand, Cx43 have a unitary conductance of 120pS (30) but they are both pH and voltage dependent(31).

A homomeric hemichannel is composed of only one type of connexin protein whereas a heterotypic hemichannel is made up of more than one type of connexin protein(32).



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Figure 1.3) Schematic model illustrating assembly of connexins into gap junctions. The connexin protein creates four membrane domains (m1–m4) with two extracellular loops (el 1+2), one cytoplasmic loop (cl), and the N and C termini exposed to the cytoplasm.(33)

1.6.2) Effect of intercellular coupling on reentry:

Under physiological conditions, a single cardiomyocyte in the adult myocardium is electrically coupled to an average of 11 adjacent cells with gap junctions being predominantly localized at the intercalated discs at the ends of the rod shaped cells(34). Both reduced coupling and heterogeneous coupling have been shown to cause arrhythmias via reentry. Reduced coupling slows down the propagation of the impulse while heterogeneous coupling creates unidirectional blocks.

As cells become less coupled, there is greater confinement of depolarizing current to the depolarizing cell with less electrotonic load and axial flow of charge to the downstream cells. This results in decreased conduction velocity of the impulse(34). Several theoretical (35-37) and experimental studies (38-40) have suggested that reduced coupling can cause major reductions in velocity before conduction block and that velocity in the context of reduced coupling can potentially be much slower than the velocity in the context of reduced excitability(41). Theoretical studies have shown that uncoupling can reduce conduction velocity to an extremely low value of 0.26 cm/s before conduction fails (41). Reduced coupling, not reduced excitability, causes a dramatic change in the conduction velocity, and this has been proved in theoretical models(41). Nevertheless, a large reduction in intercellular coupling is required to cause major slowing of conduction velocity.

Mostly, conduction slowing or block occurs at particular locations within the tissue while more robust conduction is maintained at other sites. This heterogeneity of impulse conduction plays a key role in the initiation of circulating excitation and occurrence of reentrant arrhythmias(42).

Local source-to-sink relationships determine the formation of conduction heterogeneities vis-à-vis unidirectional blocks and provide conditions for the development of slow conduction and eventually reentry(42). Heterogeneous cell-to-cell coupling increases the mismatch between the current produced upstream (source) and the downstream load (sink)(42). Ubiquitous reduction in cell-to-cell coupling as seen above is resistant against the development of propagation block because of the continuous recurrence of high resistance cell-to-cell junctions while interfaces between regions of cells with different degrees of coupling produces unidirectional blocks(34). When the cells are heterogeneously coupled, the amount of upstream tissue that is necessary to produce the excitatory current is not equally matched to the amount of downstream tissue that is to be excited. Therefore the conduction velocity can propagate only in one direction but not in the opposite direction.

Modification of cell-to-cell coupling (remodeling) could occur because of changes in connexin expression or cellular distribution patterns of gap junctions.

1.6.3) Effect of intercellular coupling on propagation of focal activity:

Although underlying cellular mechanisms initiating EADs/DADs and enhanced automaticity are different, their propagation to other cells is influenced by intercellular coupling. The small depolarizing currents successfully reach their threshold and propagate as focal activity because the electrical load of the adjacent cells is low because of reduced coupling(43). Small depolarizing currents in well coupled cells may not succeed in reaching the threshold because of the electrical load of adjacent cells(43).

When coupling is reduced, less current is shunted downstream, effectively increasing the availability of charge for local depolarization, therefore causing focal activity in that region.

1.7) OXIDATIVE STRESS IN ATRIAL FIBRILLATION:

Oxidative stress is mediated by reactive oxygen species (ROS). ROS are low molecular weight molecules (superoxide radical oxygen ions, oxygen centered free radicals, peroxides) that are partially reduced derivatives of molecular oxygen. ROS that are thought to be involved in cardiac arrhythmogenesis are superoxide anion, H_2O_2 , the hydroxyl radical (OH), peroxynitrite ($ONOO^-$) and nitric oxide (44). ROS are highly reactive because of the presence of unpaired electrons in their outer shells, and therefore, they are capable of oxidizing multiple biomolecules like protein, deoxyribonucleic acid, ribonucleic acid, lipids.

Clinical studies done in patients suffering from atrial fibrillation have reported marked increases in the pro oxidant genes as well as a decrease in antioxidant genes (45). Oxidative stress is associated with both persistent and permanent types of atrial fibrillation (46;47). Various types of ROS have been associated with atrial fibrillation. Uncoupling nitric oxide synthase (NOS) in cells isolated from right atrial appendages of patients suffering from atrial fibrillation caused an increase in the levels of superoxide molecules(48). The same study reported an increase in NADPH oxidase activity, which is another source of ROS (48). Dudley et al. showed that atrial fibrillation increased the production of superoxide in the left atrium and left atrial appendage(49).

1.8) A POSSIBLE MEDIATOR OF THE EFFECT OF OXIDATIVE STRESS ON GAP JUNCTION REMODELING:

The interaction between the carboxyl terminal domain of Cx43 and the cytoplasmic loop is facilitated by an interaction of the nonreceptor tyrosine kinase c-Src with the carboxyl terminal of the Cx43, which causes a loss of interaction of Cx43 with the scaffolding protein Zonula Occludens-1 (ZO 1)(50). Myocyte culture studies indicate that transfection of the N-terminal domain of ZO-1 caused a dominant negative effect, inhibiting Cx43 from binding to the full-length ZO-1, resulting in internalization of Cx43 and indicating that ZO-1 was playing a scaffolding and stabilizing role in localization of Cx43(50). In an animal model of myocardial infarction, the up-regulation of c-Src and an increase in the level of phosphorylated Tyr 416 c-Src (the active form of c-Src) result in the down-regulation of Cx43 by competition between phosphorylated c-Src and Cx43 for a binding site at zonula occludens-1, an intercalated disk scaffolding protein(50). In a transgenic mouse model of cardiac-restricted overexpression of angiotensin-converting enzyme (ACE8/8), inhibition of c-Src tyrosine kinase prevents angiotensin II-mediated connexin-43 remodeling(51). In the same animal model (ACE8/8), a mitochondrial antioxidant, mito-TEMPO, prevented connexin remodeling and sudden cardiac death(75). Additionally, in a manganese superoxide dismutase (MnSOD) knock out mouse model, which showed 66% increase in mito-ROS when compared to its normal, PP1, a c-Src Inhibitor, prevented connexin remodeling(74).

1.9) DIFFERENT ANIMAL MODELS OF ATRIAL FIBRILLATION:

Until the mid-1990s, virtually all experimental studies of AF were performed in animals with normal hearts, with vagal nerve stimulation or acetylcholine infusion often used when sustained AF was needed(52). Nevertheless, AF has been associated with numerous organic diseases such as congestive heart failure (CHF), mitral valve disease; coronary artery disease, and hypertensive heart disease. Hence animal models (Table 1) with the underlying heart disease can give a better understanding of atrial fibrillation and ability to treat this challenging rhythm disorder.

2) RATIONALE OF THIS STUDY:

2.1) Why Connexins?

Measurements of connexin quantity and distribution have been confined to small pieces of the right atrium in most studies. Studies have reported an increase in Cx43(53) and Cx40(54;55), a decrease in Cx43(55) and Cx40(55;56) or no change in Cx43(57) and Cx40(58). Since there have been reports of heterogeneous modeling,(55) it is possible that different regions may show quantitative differences in connexin remodeling. If this is seen, these heterogeneities in connexin distribution could cause heterogeneous electrophysiological properties that could be important in causing atrial fibrillation or fibrillatory conduction.

MODEL	SPECIES	CLINICAL PARADIGM
Sterile pericarditis	Dog	Post cardiac surgery
Atrial tachycardia remodeling	Dogs, goats	Atrial tachycardia, AF remodeling
CHF-related AF	Dogs, rats	CHF, sick sinus syndrome,
Acute atrial ischemia	Dog	Acute MI and chronic coronary disease
Atrial volume overload	Rabbit, dog, sheep	Acute severe volume overload
Mitral regurgitation	Dogs	Mitral valve disease

Table 1. Animal Models of AF in the Presence of Cardiac Pathology(52).

2.2) Why Left Atrium?

Haissaguerre et al. showed that pulmonary veins are the major source of rapid focal activities that initiate paroxysmal AF and radiofrequency ablation of these sites terminates AF(27). Several studies have shown that together with the PVs, many extra-PV areas may be the source of initiation and maintenance of AF(59). The most common sites are the superior vena cava, ligament of Marshall, coronary sinus, crista terminalis, and left atrial (LA) posterior wall(59). The left atrial appendage (LAA) is an underreported site of initiation of AF(60). The LAA appears to be responsible for recurrence of AF/tachycardia in at least 27% of patients presenting for repeat procedures(59).

ERP shortening was more prominent in the left atrium (LA) than in the right atrium,(RA) and AF inducibility was always higher in the LA than in RA(61;62). Also, very few studies have reported connexin quantifications in the LA. Keeping these in mind, it made sense to study the posterior left atrium and left atrial appendage.

A handful of studies have done connexin measurements in regions indicated to be a possible source of AF(31). Therefore, it was rational to study the expression levels of gap junctional proteins, connexins in different regions of LA during AF because they can give information regarding intercellular coupling. Regional differences in their expression (Posterior Left Atrium and Left Atrial Appendage) may provide additional information regarding the initiation and maintenance of AF. A congestive heart failure model (CHF) was used to study atrial fibrillation as many studies have shown that these two frequently co-exist and also serves as an independent risk factor of AF(63-67).

3) MATERIALS AND METHODS:

Animals were bought and housed at Northwestern University. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). Approval for use of purpose-bred dogs (hounds) was obtained from the Institutional Animal Care and Use Committee. All electrophysiological studies, cell and tissue isolation were performed at Northwestern University. Protein expression and ROS measurements were done at the University of Illinois, Chicago.

3.1) Congestive Heart Failure (CHF) Animal Model:

Sixteen purpose-bred hound dogs around 6-12 months of age were implanted with a pacemaker using sterile surgery. The details of the pacing model were described previously (68;69). The right ventricle was paced at 240 bpm for three weeks, and the left ventricle function was assessed as previously described (70). Eight nonpaced dogs between 8-12 months of age were used as controls.

3.2) Atrial Fibrillation Sustainability:

AF induction was attempted in the Left Atrial Appendage and Posterior Left Atrium during baseline with atropine and with double autonomic blockade. The LAA and PLA region were burst paced at a cycle length of 180 ms to 100 ms (with 10 ms decrements) for 10 seconds at each cycle length as previously described (70).

3.3) Western Blotting:

Snap frozen tissues from PLA and LAA regions of dogs were homogenized using cell lysis buffer (cell signaling, #9803) containing protease phosphatase inhibitor (Thermo scientific, #78442). 10 µg protein samples containing 1:1 Laemmli sample buffer (Bio-rad, # 161-0737) were separated using 4-20% SDS gel electrophoresis and then transferred to nitrocellulose membranes. The membranes were then incubated with primary antibodies against connexin43 (Dilution-1:5000, cell signaling, #3512), connexin40 (Dilution-1:5000, Santa Cruz Biotechnology, #20464), and phosphorylated c-Src (Tyr416) (Dilution-1:1000, Millipore, #05-677). Following incubation with the primary antibodies, the membranes were incubated with secondary antibodies conjugated to HRP. Protein bands were detected using ECL

chemiluminescence kit (Thermo scientific, #32132). Membranes were stripped to reblot for β -actin (Dilution 1:5000, Santa Cruz Biotechnology, #1616) to normalize protein expression. Analysis was done using ImageJ software to calculate band density.

3.4) Measurements of mitochondrial ROS production using confocal microscopy:

Cardiomyocytes were isolated as previously described (71). They were plated onto laminin (3 $\mu\text{g}/\text{cm}^2$, BD Biosciences, #354329) glass bottom microwell dishes (3.5 mm petri dish, 1.4 cm microwell) and then were treated with MitoSOX red (5 $\mu\text{mol}/\text{l}$, Invitrogen, M36008#) in MEM medium (containing 1% Penicillin/streptomycin, 1% Insulin-Transferrin-Selenium (ITS), 1% L-glutamine, 1% bovine serum albumin) for 10 min at 37°C in CO₂ incubator. This was followed by washing them twice with the same medium. The cells were excited at 515 nm and the emitted light was measured between 520-620 nm using Zeiss confocal microscopy (Zeiss 710 LSM, 40X water immersion objective lens). The mitochondrial subcellular location of MitoSOX was confirmed by co-labeling with 50 nmol/L MitoTracker Green FM (Invitrogen, M-7514) (excitation/emission: 490/516 nm). Fluorescence was measured every 5 min on at least 8 different areas on the culture dish. Images were analyzed using Image J and corrected total fluorescence (CTGF) obtained from using Image J was calculated using:

CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings) (72).

3.5) Statistical Analysis:

Data are expressed as mean \pm SEM. Statistically significant differences were calculated using Student's t test (for paired or unpaired samples). P values <0.05 were recorded as statistically significant values.

4) RESULTS:

4.1) Atrial Connexin remodeling:

4.1.1) Connexin 43 and 40 expression in PLA and LAA region:

Western blot analysis of Cx40 protein at the PLA region of CHF samples showed no significant change (3.5%, P=NS)) with respect to the control dogs (Figure 4.1B). On the other hand, Cx43 protein analysis shows a trend towards a decrease in its expression in the CHF samples (43.9%, P=NS), but the decrease was not statistically significant (Figure 4.1C). Representative blots show that there was no visible reduction in the connexin proteins in the CHF samples (Figure 4.1A).

In contrast to the findings seen in the PLA regions, Western blots shows marked reduction in both Cx40 as well as Cx43 (Figure 4.2A) in the LAA region of the CHF samples when compared to their controls. Analysis of the blots showed statistically a significant decrease in Cx40 in the LAA of the CHF samples (53.06 %, P<0.001) (Figure 4.2B) and a steep significant decrease (88.43%, P<0.001) in the Cx43 expression in the LAA of the CHF samples (Figure 4.2C).

4.2) Oxidative stress:

4.2.1) Mito-ROS levels in PLA and LAA regions:

MitoSOXTM is a fluorogenic lipophilic dye that is specifically targeted to mitochondria in live cells. Oxidation of MitoSOXTM by superoxide produces red fluorescence. Mitotracker Green is a green fluorescent mitochondrial stain that localizes to mitochondria in live cells thereby serving as a labeling protein. The red fluorescence intensity in the CHF PLA (1.8 ± 0.3), (Figure 4.4B) was comparatively higher when compared to their controls (0.43 ± 0.039 , P<0.05) (Figure 4.4A). The same was observed in the LAA region of the control (0.67 ± 0.13) (Figure 4.3A) and CHF (3.19 ± 0.82 , P<0.001)) (Figure 4.3B) samples. Although both regions showed increased

mito-ROS production, it can be seen that the ROS levels in heart failure LAA (Figure 4.3C) is higher than the ROS levels in the heart failure PLA (Figure 4.4C) region by 43 %.

4.3) Src, a possible mediator of the effect of mitoROS on connexins:

4.3.1) Phospho Src levels in heart failure PLA versus heart failure LAA samples:

The phospho Src (active form of c-Src) was measured in the PLA and LAA heart failure samples. There was a significant increase in the levels of phospho c-Src in the LAA region when compared to the PLA region (Figure 4.5C).

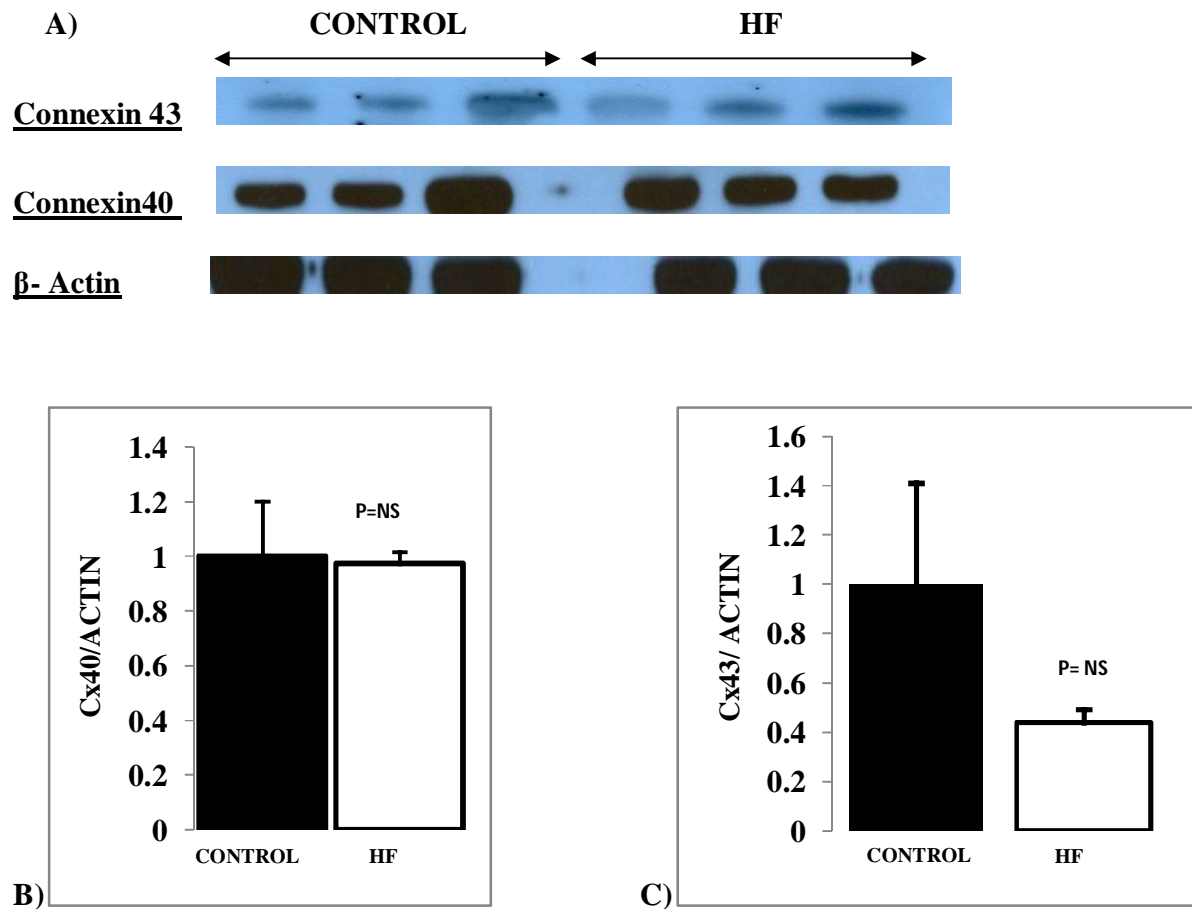


FIGURE 4.1): Connexin 40 and 43 expression in PLA of control and heart failure animals. **A**, Protein bands showing the expression of Connexin 40 and 43 in control and heart failure dogs, n=3.β- Actin was used to standardize the expression of proteins. **B**, onnexin 40 expression in Control and Heart failure, p value = NS. **C**, Connexin43 expression in control and heart failure, p value =NS.

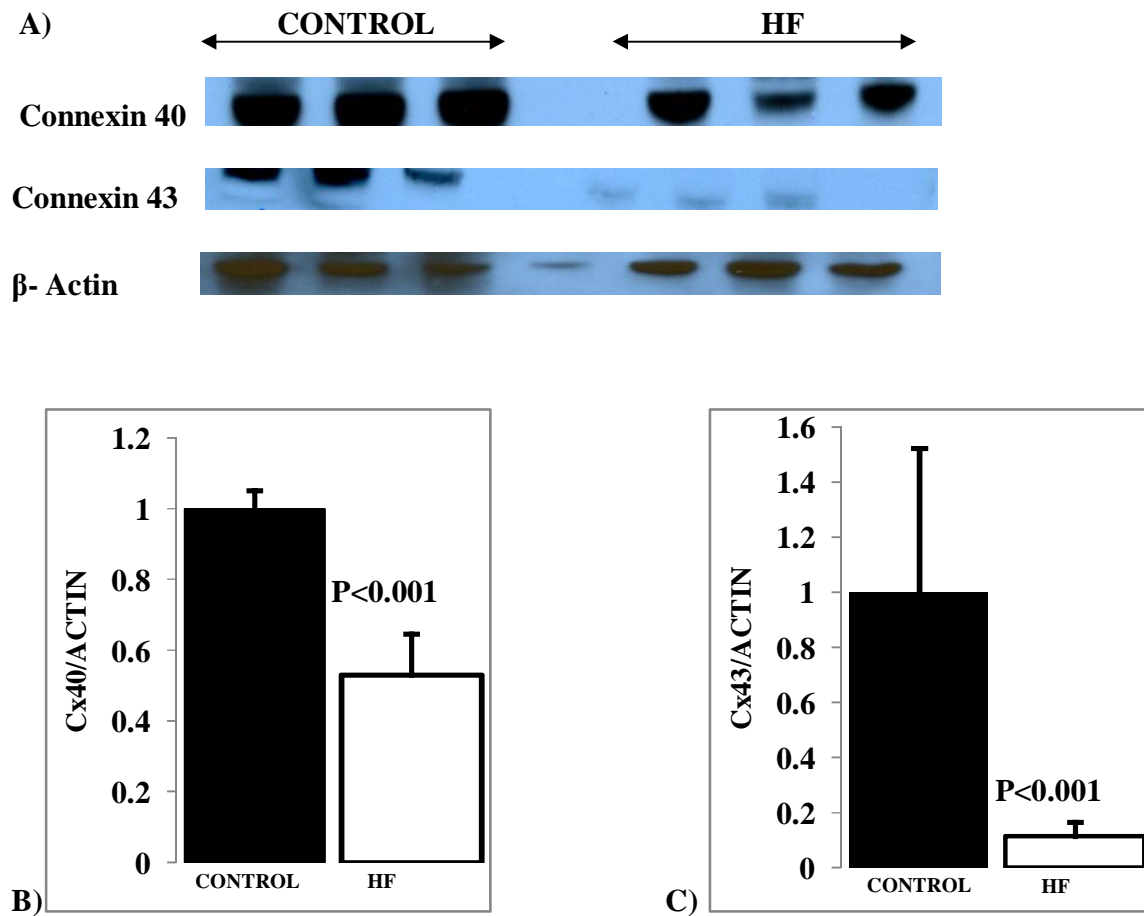


Figure 4.2) Connexin 40 and 43 expression in LAA in control and heart failure dogs. Protein bands showing the expression of Connexin 40 and 43 in control and heart failure dogs, n=3. β - Actin was used to standardize the expression of proteins. **B**, Connexin 40 expression in Control and Heart failure, p value <0.001. **C**, Connexin43 expression in control and heart failure, p <0.001.

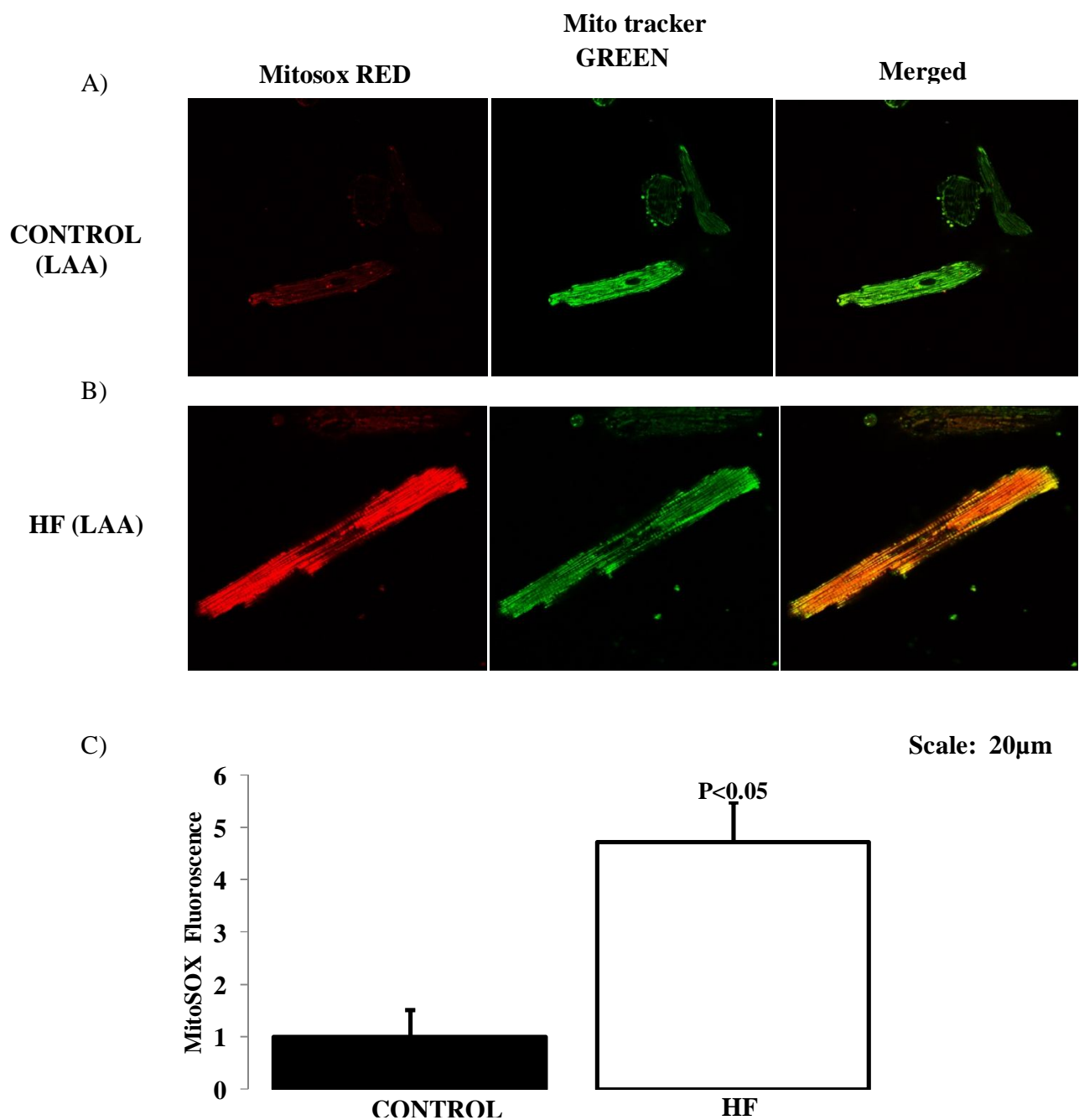
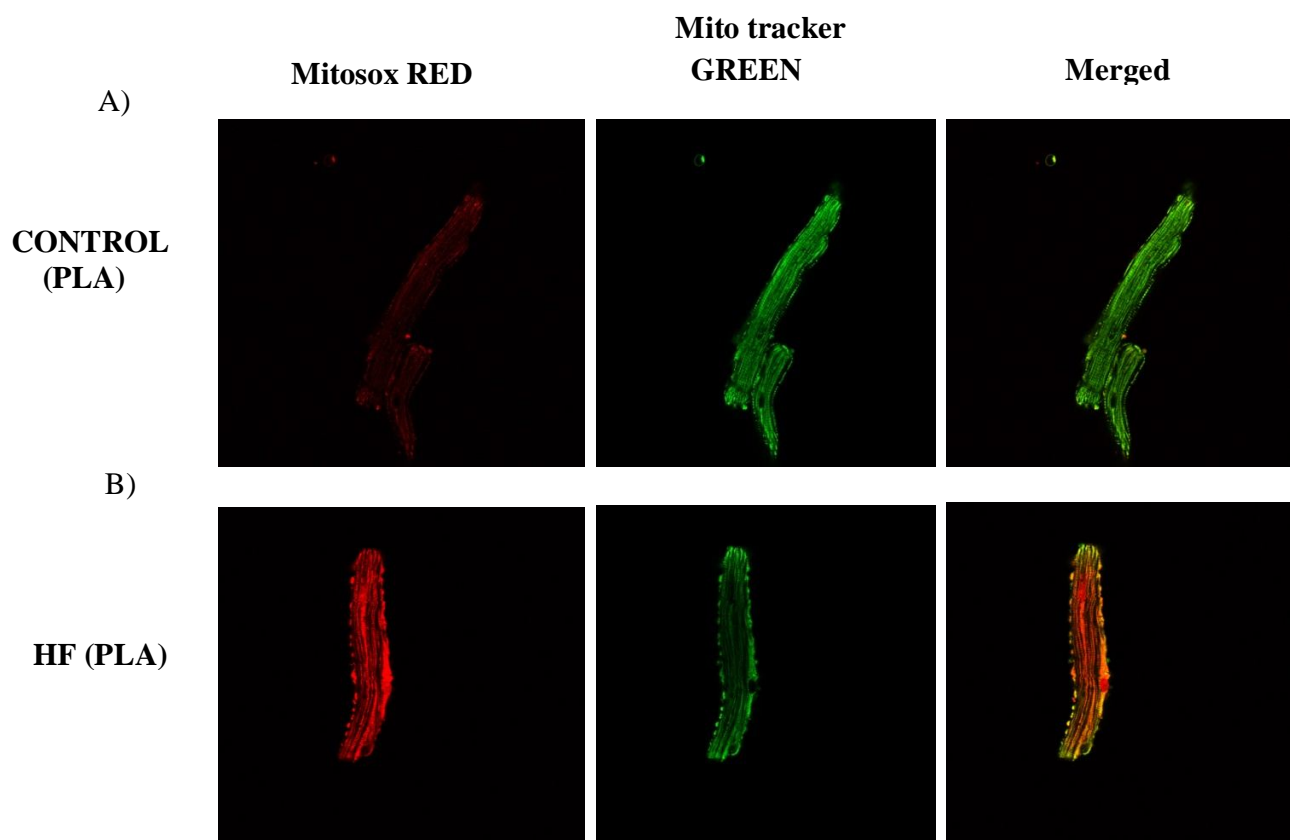


FIGURE 4.3) Mitochondrial ROS(mito-ROS) measurements in LAA Control versus Heart Failure. MitoSOX Red (Left panels) co localized with MitoTracker Green (middle panel) in merged images (Right Panel), indicating high superoxide anion concentration in mitochondria of HF LAA. Scale bar is 20 µm; **A**, Images represent a single cardiomyocyte from the control LAA sample. **B**, Images represent a single cardiomyocyte from the heart failure LAA sample. **C**, Level of red fluorescence intensity in control versus heart failure samples.



Scale: 20μm

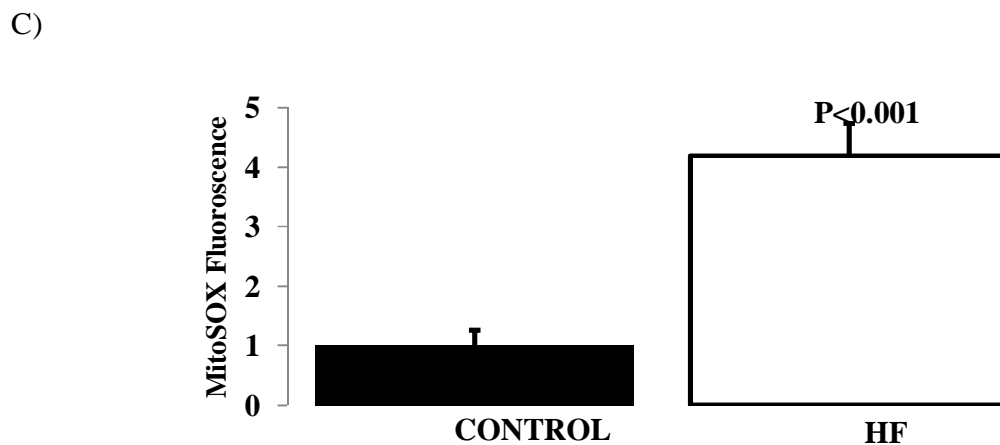


FIGURE 4.4) Mitochondrial ROS (mito-ROS) measurements in PLA control versus heart failure. MitoSOX Red (Left panels) co-localized with MitoTracker Green (middle panel) in merged images (Right Panel), indicating high superoxide anion concentration in mitochondria of HF PLA. Intensity of redness correlates to the level of mito-ROS present in the cell. Scale bar is 20 μm; **A**, Images represent a single cardiomyocyte from the control PLA sample. **B**, Images represent a single cardiomyocyte from the heart failure PLA sample. **C**, Level of red fluorescence intensity in control versus heart failure samples.

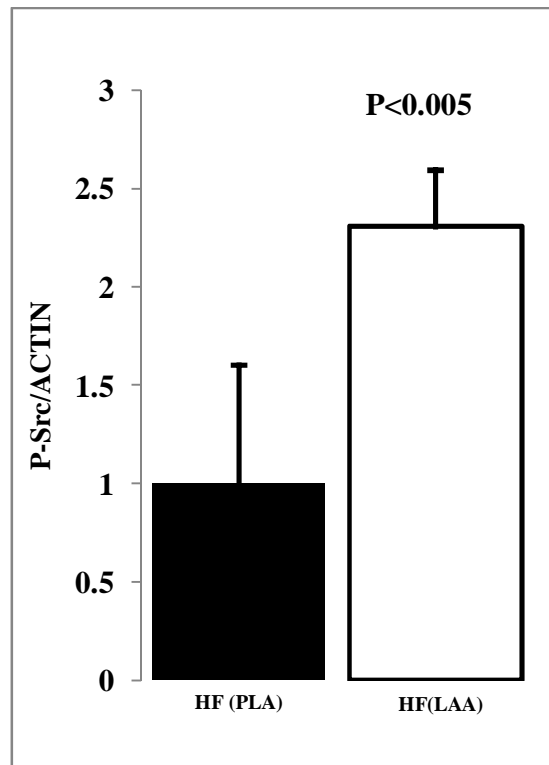
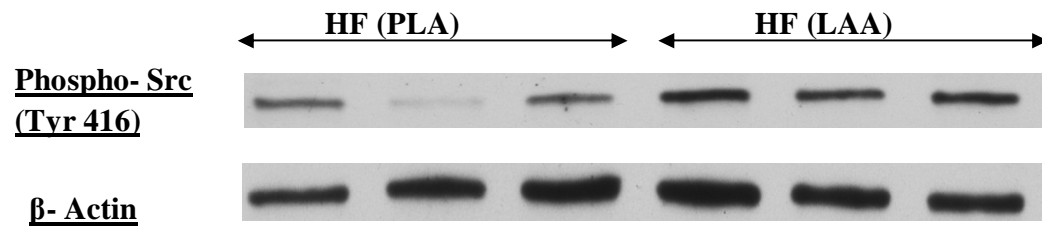


Figure 4.5): Phospho Src levels in Heart Failure - PLA versus LAA tissue samples. A, shows the density bands of Phospho src expression in these tissue sample. They were normalized to the actin levels in these tissues. **B,** Western blot data in these samples. $P < 0.005$.

5) DISCUSSION:

This study compared the expression levels of the main atrial connexins (40 and 43) in the posterior left atrium and left atrial appendage. . Since ROS was associated with AF, levels of ROS were also studied. Src was studied because Src could be a possible mediator of the effect of ROS on connexin. This study found a correlation between elevated mito-ROS, activated Src, and reduced Cx43 in the LAA. Also, this study showed that there are regional differences in the expression of connexin in the left atrium of the CHF heart that could provide substrate for slow conduction and unidirectional blocks.

This model of CHF-induced AF showed that there regional differences in the connexin expression in the left atrium and that their expression levels were inversely correlative with levels of oxidative stress and activation of c-Src present in these regions. These findings are consistent with previous studies done in mouse models of angiotensin-converting-enzyme over expressed (ACE 8/8) (75) and manganese superoxide dismutase (MnSOD) knockout mice (74), where ROS levels and activated Src are inversely correlative with the level of connexins.

Different animal and human models have studied the levels of connexins during AF. All atrial tachypaced goat models have shown no change in Cx43 in the left atrial appendage (LAA), right atrial appendage (RAA) and the right atrium, but there are reports of unchanged and decreased Cx40 (Table 2) in the same models. In our model, we reported decreased connexins in the LAA. This discrepancy could have resulted from the different animal model used or the fact that atrial tachypaced (ATP) models are not associated with CHF. Suggesting the latter, our findings were consistent with another study that checked for connexin43 and connexin40 expression levels in

the left atrial wall in the same canine tachypaced CHF model(73). Other models of AF in humans and pigs have reported increased connexin, decreased connexin or no change in connexin expression (Table 2).

The regional differences in the connexins found in LAA and PLA can be explained by the variations in Src activation in these regions. Activation of c-Src was found to be higher in the CHF LAA region than the CHF PLA region. Along with this activation, a higher level of ROS was found in the CHF LAA region. Putting these together and from the findings in this study, it is safe to say that c-Src could be a possible mediator of the effect of ROS on connexin43. To confirm this finding, it was previously shown that inhibition of c-Src tyrosine kinase prevents connexin43 remodeling in the ventricles of a mouse model. Nevertheless, Cx40 regulation appears different than that of Cx43.

	AF model	Connexin Expression		Tissue Area Used to study connexin expression	Author & Year
		CX40	CX43		
GOAT	Atrial Tachypacing (ATP) Induced persistent AF	→	→	Left Atrial Appendage (LAA) and Right Atrial Appendage (RAA)	Van-der Velden et al. (1998)
	Atrial Tachypacing (ATP) Induced persistent AF	↓	→	LAA and RAA	Van-der Velden et al. (2000)
	Atrial Tachypacing (ATP) Induced AF	↓	→	LAA and RAA	Ausma et al. (2003)
	Atrial Tachypacing (ATP) Induced paroxysmal AF	→	→	Right atrial myocardium	Neuberger et al. (2010)
DOG	Sterile pericarditis induced AF	↓	↓	Right atrium	Ryu et al. (2007)
	Congestive heart failure induced AF	→	→	Left Atrial wall	Burstein et al. (2009)
HUMAN	Post operative AF (patients with coronary artery disease (CAD))	↑	→	RAA	Dupont et al. (2001)
	Chronic AF > 1 year (patients undergoing mini maze procedure)	↓	↓	Right atrium and RAA	Kostin et al. (2002)
	Chronic AF> 5 months(patients with mitral valve disease)	↓	→	Right Atrium	Nao et al. (2003)
	Permanent AF > 3 months	→	↑	Left Atrium	Adam et al. (2010)
	Persistent AF> 3 months	↓	→	RAA	Wilhelm et al. (2006)

Table 2: Connexin Expression studies done using different clinical and experimental animal models of Atrial Fibrillation.

Based on the Hunter, McNaughton and Noble model of propagation in excitable cells, the relationship between conduction velocity and gap junction conductance can be derived.

$$\theta \propto a^{1/2} R_i^{-1/2} C_m^{-5/8} \tau_m^{-3/8} g_{Na}^{1/8} \dots\dots (1)$$

Where θ = conduction velocity, a = radius of the cell, R_i is the intracellular resistivity, C_m is the membrane capacitance, τ_m is the membrane time constant and g_{Na} is sodium channel conductance. From Eq (1) it is clear that conduction velocity is inversely proportional to the square root of intracellular resistivity. R_i is nothing but the sum of cytoplasmic resistance (R_c) and junctional resistance (R_j) and can be written as,

$$R_i = R_c + R_j \dots\dots (2)$$

Assuming that the other parameters in Eq (1) are constant and substituting Eq (2) in Eq(1) we get,

$$\theta \propto \frac{1}{\sqrt[2]{R_c + R_j}} \dots\dots\dots (3)$$

The cytoplasmic resistance for a particular cell is constant. Assuming a value of 100 Ω for the cytoplasmic resistance Eq (3) becomes,

$$\theta \propto \frac{1}{\sqrt[2]{100 + R_j}} \dots\dots\dots (4)$$

We know that resistance is the reciprocal of conductance. Eq (4) becomes,

$$\theta \propto \sqrt{\frac{g_j}{100g_j + 1}} \dots\dots\dots (5)$$

Where, g_j is gap junction conductance.

Gap junctional conductance can be expressed by the following terms,

$$g_j = N\gamma_0 \dots\dots (6)$$

Where, γ_0 is the unitary conductance of one gap junction channel and N is the number of gap junction channels.

From Equation (5) and Equation (6), it is clear that conduction velocity is directly proportional to the number of gap channels. Therefore, a decrease in the number of gap channels should decrease the conduction velocity of the impulse. This study shows that there is greater reduction in connexin protein at the CHF LAA region which could mean that the conduction velocity is slower in CHF LAA. As discussed before, slow conduction velocity provides substrate for reentrant mechanism.

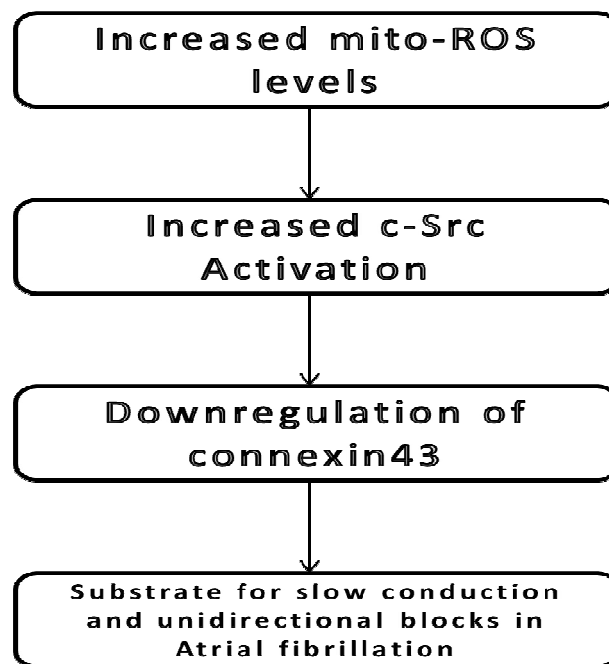


Figure1.5) : A flowchart of my findings.

6) LIMITATIONS:

This study does not confirm whether the decrease in connexins causes reduction in conduction velocity. The connexin expression profiles of the right atria were not studied. Connexin quantity cannot be equated with structural and/or functional gap junctions. Connexin proteins may also locate in the membrane without forming structural gap junctions.

7) CONCLUSIONS:

By using an animal model of Congestive Heart Failure associated with AF, changes in connexin expression in the left atria have been studied. CHF LAA has been shown to have higher oxidative stress, increased c-Src activation, and reduced Cx43. It is evident from the results that a fibrillating atrium undergoes connexin remodeling associated with increased oxidative stress and heterogeneous c- Src activation. Nevertheless, my findings only suggest a possible mechanism behind the reduction in conduction velocity. A reduction in the connexin quantity could cause a reduction in the gap junction conductance. This reduction in gap junction conductance could also reduce conduction velocity thus providing a substrate for the fibrillation to perpetuate. Although Cx43 and Cx40 quantifications in the left atria have been measured in the past, no study has reported any decrease in the level of Cx43 and Cx40 in the LAA of heart failure dogs. Also, the role of oxidative stress in gap junction remodeling has never been established in the atria.

8) FUTURE DIRECTIONS:

Conduction velocity in the PLA and LAA region must be studied in order to show if reduced intercellular coupling decreases conduction velocity. Quantification of structural gap junctions at the intercalated discs must be done in order to check if the reduction in the connexin quantity correlates to the reduction in the structural gap junctions.

We need to establish a cause and effect relationship with the levels of ROS, Src activation, and connexin levels by checking if antioxidant treatments targeting mitochondrial ROS and Src inhibitors prevent Cx43 remodeling and atrial fibrillation.

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