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Electrophysiological Dynamics and Underlying Mechanisms of Long Duration Ventricular Fibrillation

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ELECTROPHYSIOLOGICAL DYNAMICS AND UNDERLYING MECHANISMS OF LONG DURATION VENTRICULAR FIBRILLATION

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A THESIS

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ABSTRACT

Sudden cardiac death claims more than 300,000 lives per year in the US alone. Because it frequently goes unwitnessed, the underlying ventricular fibrillation (VF) may persist for well over 5 minutes (long duration VF, or LDVF) before attempts at defibrillation, and successful defibrillation is often followed by post-shock arrhythmias and refibrillation. The development of novel and effective therapies for LDVF, which represents a significant unmet medical need, remains largely hindered by a relatively poor understanding of the arrhythmogenic onset and maintenance of LDVF, as well as its deleterious sequelae in the context of the whole heart. Prolonged VF has been associated with development of a transmural activation gradient, with marked slowing of epicardial activation, the basis of which remains undetermined. Dysregulation of intracellular calcium *per se* may contribute to persistence of LDVF and increased post-shock arrhythmias -- whether through enhanced triggered activity or altered conduction – as well as altered contractile dysfunction post defibrillation. In these studies, we explored changes in epicardial activation and intracellular calcium, and their relationship to each other during LDVF. We hypothesized that LDVF is associated with slow epicardial activation that is related to altered electrical and intracellular calcium dynamics. A stable model of LDVF was developed in the rabbit heart, since most studies of LDVF have been limited to large animal (canine and porcine) models. Epicardial action potentials and intracellular calcium transients were optically mapped simultaneously, and

complementary intramural plunge electrodes were incorporated for recording transmural activation. At 4 min of VF, a transmural activation gradient developed with significant increases in cycle length (CL), diastolic interval (DI), calcium transient (CaT) duration (CaD), and CaT recovery time. The results of these studies suggest that impaired calcium handling does not play a major role in the initial slowing of epicardial activation during LDVF, but may contribute to prolonged VF maintenance and post-shock arrhythmias. These studies provide important insights into the biochemical mechanisms underlying LDVF and the foundation for developing and testing of novel therapeutic approaches to the treatment of LDVF.

Keywords: ventricular fibrillation, optical mapping, action potential, intracellular calcium

DEDICATION

I dedicate this work to my parents, for their endless love, support, and encouragement in everything I do. I could not have completed this work without them.

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I gratefully acknowledge the members of staff at the Cardiac Rhythm Management Laboratory who assisted in the execution of these studies, including Frank Vance, Sharon Melnick, and Shannon Salter, who provided necessary surgical assistance during animal studies. I extend my thanks to Joseph Barchue for his assistance with tissue collection. I would also like to thank Dennis Rollins for his assistance and training on the use of the electrical mapping system and data acquisition software.

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ELECTROPHYSIOLOGICAL DYNAMICS AND INTRACELLULAR CALCIUM HANDLING DURING LONG DURATION VENTRICULAR FIBRILLATION IN ISOLATED RABBIT HEARTS

LIST OF ABBREVIATIONS

AP	action potential
APD	action potential duration
APD_{60}	action potential duration to 60% recovery
CaD	calcium transient duration
CaD_{60}	calcium transient duration to 60% recovery
CaT	calcium transient
CL	cycle length
CMOS	complementary metal-oxide-semiconductor
Cx43	connexin 43
DAD	delayed afterdepolarization
DI	diastolic interval
EAD	early afterdepolarization
ECG	electrocardiogram
endo	endocardium
epi	epicardium
g_j	gap junctional conductance
K_{ATP}	ATP-sensitive potassium channel
LDVF	long duration ventricular fibrillation
LED	light emitting diode

LV	left ventricle
OOHCA	out of hospital cardiac arrest
RV	right ventricle
SCR	spontaneous calcium release
SR	sarcoplasmic reticulum
VF	ventricular fibrillation

INTRODUCTION

Clinical Significance

Sudden cardiac death from out-of-hospital cardiac arrest (OOHCA) is most commonly due to ventricular fibrillation (VF) and leads to over 300,000 deaths per year in the US alone. Survival rates from OOHCA are dismal, running as low as 8%.[1] Two contributors are largely responsible for this poor prognosis: many OOHCA's are unwitnessed, and attempts to defibrillate long duration VF (LDVF – VF lasting 5 minutes or longer) are frequently unsuccessful, although survival with neurologic recovery is possible even with LDVF lasting up to 10 minutes.[2] Approaches to treatment of LDVF include CPR, correction of acidosis, catecholamines, defibrillation, and, more recently, therapeutic hypothermia. None of these treatments have had a significant impact on the extremely high mortality of LDVF, due in part to contractile dysfunction and reperfusion injury associated with prolonged ischemia.[2,3] Thus, there is a need for novel approaches to therapy of LDVF. Generating novel approaches critical to effective therapy requires a thorough understanding of the electrophysiological and biochemical mechanisms underlying LDVF.

Transmural Gradient of Activation

The Ideker lab at UAB first described a marked transmural gradient of activation rate during LDVF, in which endocardial activation remains fast with sharp, distinct deflections, while epicardial activation becomes slow and indistinct by 5-7 minutes of

LDVF.[4] Further studies have confirmed the development of a transmural activation gradient during LDVF in canine[5-7] and human[8] hearts, though the basis of slow epicardial activation has yet to be determined and multiple factors may contribute. Studies conducted by Kong et al. at UAB have shown the development of a transmural activation gradient concurrent with desynchronization between action potentials and intracellular calcium transients. The rate at which desynchronization occurred varied transmurally and was associated with prolonged calcium transients, indicating calcium cycling impairment.[5] These findings suggest a potential role of impaired calcium handling in the development of a transmural activation gradient during LDVF and the maintenance of prolonged VF.

Intracellular Calcium and Altered Cell Coupling

VF has been associated with calcium cycling impairment, intracellular calcium overload,[9,10] and dissociation of membrane potential and intracellular calcium transients,[11] which has been linked to post-defibrillation contractile dysfunction, reperfusion injury, and refibrillation.[2,3,10] Studies in a canine model have shown the development of a transmural activation gradient concurrent with desynchronization between action potentials and intracellular calcium transients, as well as prolonged calcium transients, indicating calcium cycling impairment.[5] Furthermore, sustained elevated intracellular calcium has been connected to decreased cell coupling that is thought to be due in part to the dephosphorylation of the gap junction protein connexin 43 (Cx43), though the relationship between intracellular calcium and Cx43 has not been fully elucidated.[12,13] Heterogeneous localization and dephosphorylation of Cx43 may play an

important role in VF by contributing to slow conduction and altered cell coupling. [14] Cx43 is a phosphoprotein that can be phosphorylated by a number of protein kinases (e.g. protein kinase C, MAP kinase, src)[15-22] and dephosphorylated by protein phosphatases (which may be activated by increased intracellular calcium[12,13]) leading to altered gap junctional conductance (g_j).[23-25]

Aims

The mechanisms underlying long duration ventricular fibrillation remain poorly understood. A majority of the studies conducted in VF to date have focused on short duration VF (usually less than 1 min). Of the limited studies in LDVF, many have used perfused VF, which may have restricted clinical relevance, though a few studies have begun to examine nonperfused LDVF. Studies in LDVF have primarily been conducted in larger animal models, such as dog and pig, likely due to the critical mass needed to sustain VF. However, smaller animal models provide an attractive option for higher resolution mapping and pharmacological studies that may be more challenging in larger models. Furthermore, a rabbit model of LDVF may be studied in parallel with heart failure rabbit models, which have been developed in the Pogwizd lab at UAB.

Transmural activation and calcium dynamics have not been well described in the intact rabbit model of LDVF. Some previous studies have investigated the roles of altered activation and calcium handling associated with VF, but none have thoroughly examined those dynamics in the LDVF rabbit model. Furthermore, most studies exploring the role of calcium in VF have either been conducted in short duration VF,[11,26] or after defibrillation.[27,28] Relatively few studies have investigated calcium dynamics during

prolonged VF in an intact heart.[5] Understanding the biochemical mechanisms underlying LDVF is essential for the development of novel therapies to improve patient outcomes.

In these studies, we hypothesized that LDVF is associated with a slow epicardial activation rate that is related to altered ventricular action potential and calcium dynamics. We intended to test our hypothesis using an isolated rabbit heart model of LDVF.

Our first aim was to develop an *in vitro* rabbit model of LDVF. Few studies have assessed transmural activation or calcium dynamics in the rabbit heart during VF. This work will therefore contribute significantly to our understanding of the mechanisms underlying prolonged VF in a novel animal model.

Our second aim was to assess epicardial action potential and intracellular calcium dynamics and transmural activation during LDVF. Most VF studies conducted in intact heart models have utilized either transmural mapping with electrodes or optical mapping of the epicardium, but not both. Our unique approach combines high-resolution optical mapping of both epicardial transmembrane potential and intracellular calcium (using a newly developed dual camera imaging system) with simultaneous transmural recordings using plunge needle electrodes. The addition of plunge needles also allows for continuous recording throughout VF, whereas optical mapping systems typically only allow for brief, periodic recordings due to the potential for dye photo-bleaching. This study combines these approaches to investigate unexplored areas and provide important insights into the maintenance of LDVF.

Development of an *in vitro* Rabbit Model of LDVF

To perform these studies, it was necessary to induce stable VF in the rabbit heart that could be sustained for >5 min. Electrical induction of VF in the isolated rabbit heart was first attempted by burst pacing the left ventricle (LV), but VF was frequently not induced or sustained. Burst pacing the right ventricle (RV) resulted in significantly longer VF durations (Figure 1), although spontaneous termination still occurred.

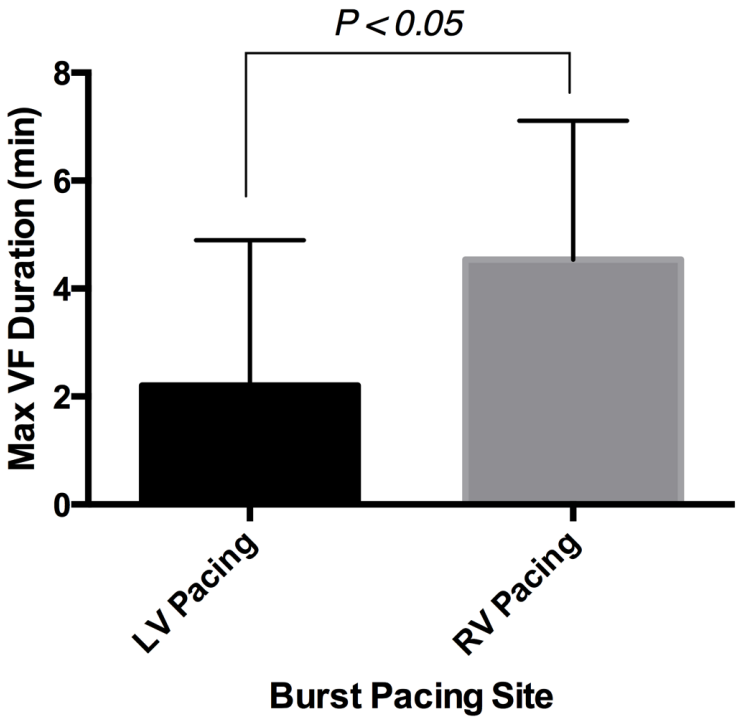

Figure 1: VF Duration by Pacing Site. The maximum duration of VF is compared between hearts paced from electrodes placed in the LV (n=10) vs RV (n=13). VF duration is significantly longer in hearts paced from the RV ($p<0.05$).

Superfusate oxygenation levels and the use of the electromechanical uncoupler blebbistatin were also evaluated as potential factors affecting VF duration. In a separate

set of studies, the superfusate in the tissue bath surrounding the heart was exchanged with a nitrogen-saturated solution during VF to reduce available oxygen (Figure 2). In another set of studies, blebbistatin was excluded to assess its effects on VF duration (Figure 3). Neither the superfusate oxygen levels nor the use of blebbistatin had a significant impact on VF duration. We therefore continued the use of blebbistatin, and did not add N_2 to the superfusate.

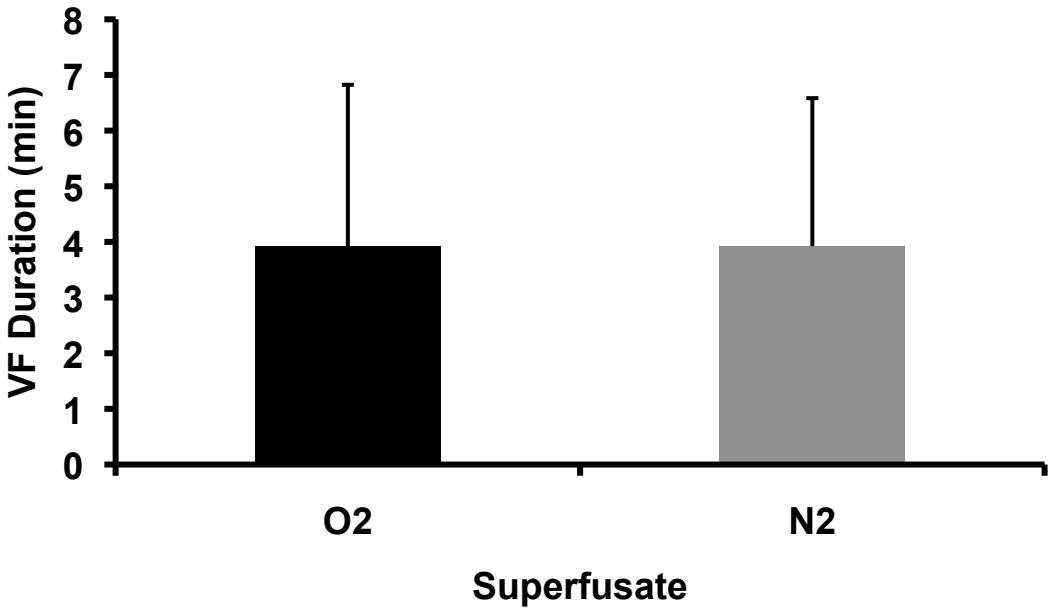

Figure 2: VF Duration by Superfusate. VF duration is compared between hearts submerged in an oxygenated superfusate (n=13) vs. hearts submerged in a nitrogen-saturated solution (n=13). Superfusate oxygenation has no significant effect on VF duration.

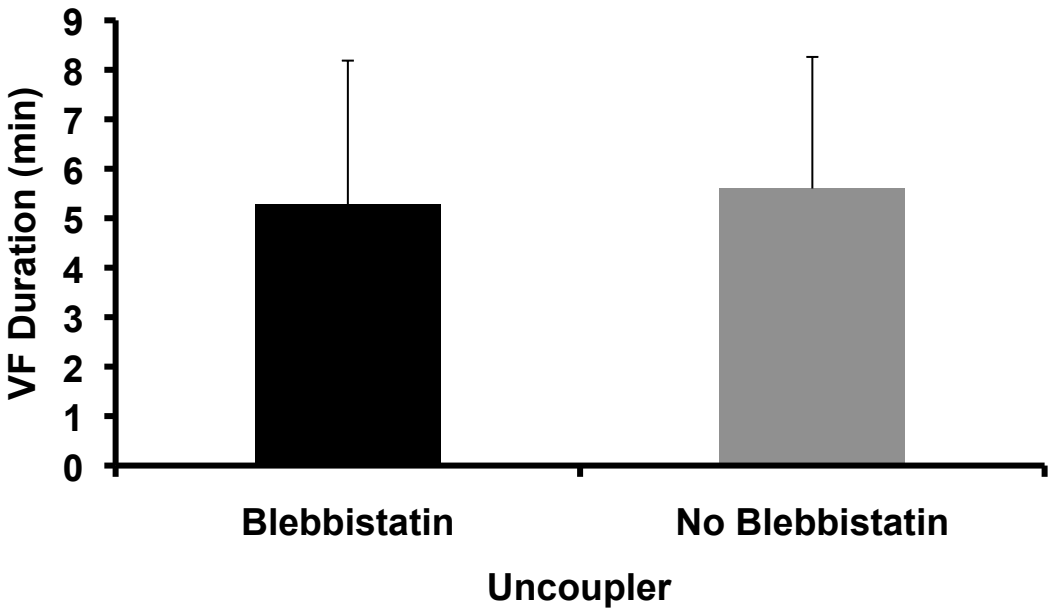

Figure 3: VF Duration by Uncoupler. VF duration was compared between hearts that were electromechanically uncoupled using blebbistatin (n = 7) vs. hearts that were not uncoupled with blebbistatin (n=5). The use of blebbistatin had no significant effect on VF duration.

ELECTROPHYSIOLOGICAL DYNAMICS AND INTRACELLULAR CALCIUM HANDLING DURING LONG DURATION VENTRICULAR FIBRILLATION IN ISOLATED RABBIT HEARTS

by

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Format adapted for thesis

ABSTRACT

Sudden cardiac death is frequently due to ventricular fibrillation (VF). Current treatments for long duration VF (LDVF) ≥5 min are largely unsuccessful, and improvements in therapies have been hindered by a lack of knowledge regarding the underlying mechanisms of LDVF maintenance. The development of a transmural gradient of activation during LDVF has been described in canine and human hearts, with a marked decrease in the epicardial activation rate. The mechanism underlying slow epicardial activation is not known. Decreased cell coupling could contribute to slow activation, and dysregulation of intracellular calcium cycling may play a role in altered cell coupling. The relationship between epicardial electrical activity and intracellular calcium cycling has not been well characterized during LDVF. These studies use combined optical and electrical mapping to assess epicardial and transmural activation and intracellular calcium dynamics during 5 min of LDVF in an isolated rabbit heart. A transmural activation gradient developed by 4 min of VF. Epicardial cycle length (CL), diastolic interval (DI), calcium transient (CaT) duration (CaD_{60}), and CaT recovery time all increased significantly at 4 min of VF. Action potentials (APs) always preceded CaTs during LDVF, although not every AP was followed by a CaT after 4 min of VF. Altered CaTs occurred concurrently with decreased epicardial activation, but not before; the time course of changes in calcium handling and the consistency of APs preceding CaTs suggests that abnormal calcium cycling and spontaneous calcium release (SCR) are likely not implicated in the initial slowing of epicardial activation in LDVF, although impaired

calcium handling may still contribute to further LDVF maintenance and post-defibrillation arrhythmias.

INTRODUCTION

Sudden cardiac death, most commonly due to ventricular fibrillation, leads to over 300,000 deaths per year in the United States alone [1]. Low survival rates for out-of-hospital cardiac arrests are largely due to delayed response times, resulting in long duration VF (LDVF – VF ≥5min) and prolonged ischemia. Current treatments have not had a significant impact on the extremely high mortality of LDVF, due in part to contractile dysfunction and reperfusion injury associated with prolonged ischemia [2,3], although survival with neurologic recovery is possible even with LDVF lasting up to 10 minutes [3]. Studies of the mechanisms of VF have primarily focused only on short duration VF, frequently lasting < 1min. The mechanisms of LDVF maintenance remain poorly understood. Novel approaches to therapy require a better understanding of the electrophysiological and biochemical mechanisms underlying LDVF.

LDVF has been associated with the development of a transmural gradient of activation in canine [4-7] and human hearts [8], with marked slowing of the epicardial activation, the basis of which remains to be determined [4-7]. Abnormal calcium handling has been implicated in numerous cardiac arrhythmias and post-ischemia reperfusion injury [2,3]; however, calcium dynamics during VF, particularly long duration VF, have not been well characterized. Dysregulation of intracellular calcium *per se* may contribute to persistence of LDVF and increased post-shock arrhythmias – whether through enhanced triggered activity or altered conduction – as well as altered contractile dysfunction post-defibrillation [2,3].

Prior studies have demonstrated increasing epicardial cycle length (CL) and diastolic interval (DI), with decreasing action potential (AP) duration (APD) during LDVF in canine [5], swine[9], and rabbit [10] hearts. A study in canine hearts simultaneously demonstrated prolonged calcium transients (CaTs) and voltage-calcium desynchronization during LDVF [5]. In the present work, we assess intracellular calcium dynamics and their potential association with altered epicardial activation during LDVF in an *in vitro* rabbit model. We hypothesized that LDVF in the rabbit heart is associated with slow epicardial activation and a transmural activation gradient that are related to changes in intracellular calcium handling.

METHODS

Heart Preparation

All animal studies complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996). The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Alabama at Birmingham. Five male, New Zealand White rabbits (>6 months, 3.5-4.5 kg) were heparinized (2 ml heparin sulfate IV, 1000 USP units/ml), sedated with ketamine (44 mg/kg), and anaesthetized with isoflurane (5%, mixed in 100% O_2), as previously described [11]. Hearts were rapidly excised, and the aorta was cannulated and Langendorff-perfused with modified Tyrode's solution (in mM: NaCl 128.2, KCl 4.7, $NaHCO_3$ 20, NaH_2PO_4 1.19, $MgCl_2$ 1.05, glucose 11.1, $CaCl_2$ 1.8, and albumin 100 mg/L) bubbled with 95% O_2-5% CO_2 and warmed to maintain physiologic pH (7.40-7.45) and temperature (36-37°C). Perfusion pressure was maintained at 50-70 mmHg with a flow rate of 20-30 ml/min. Two wire hook electrodes were inserted into the right ventricle (RV) outflow tract (2-3 mm apart) for VF induction. In some studies (n=4), a bipolar plunge needle electrode was inserted into the base of the left ventricle (LV) for transmural electrical recording.

The heart was immersed in a tissue bath of heated superfusate (36-37°C). Three electrodes mounted in the bath were used to record a continuous volume-conducted electrocardiogram (ECG) throughout the study. Hearts were allowed to stabilize for ~30 minutes in the bath before being stained with the calcium-sensitive dye Rhod-2 AM

(Biotium, 0.5mg in 0.5ml DMSO [Sigma] and 0.5 ml PowerLoad [Invitrogen]) and the voltage-sensitive dye RH237 (Biotium, 30μL of 2.52mM stock in DMSO). To prevent motion artifact in optical measurements, the electromechanical uncoupler blebbistatin (Caymen Chemicals, 20μM) was added to the perfusate and recirculated throughout the experiment.

Optical and Electrical Mapping

Excitation light was provided by an array of six green light emitting diodes (LEDs) (Luxeon Rebel, 530 nm) with bandpass excitation filters (Chroma Technology, 530/30 nm). The emitted fluorescence was collected by a tandem lens assembly (Nikon, non-AF Nikkor lenses) and separated by a dichroic mirror (Chroma Technology, 630 nm LPXR, >95% transmission). The separated voltage and calcium emission spectra passed through emission filters (Chroma Technology, 695 nm longpass and 590/33 nm, respectively) before being focused on two complementary metal-oxide-semiconductor (CMOS) detectors (RedShirt Imaging). Each CMOS detector has a 16.4 x 16.4 mm chip with 128 x 128 pixels. A magnification of 0.48X was used, allowing for a pixel dimension of 0.27 mm. All signals were sampled at 1 kHz using custom acquisition software (Redshirt Imaging).

In studies with electrical mapping, bipolar plunge needle electrodes were connected to a 256-channel mapping system. A ground electrode was placed in the tissue bath. Signals were sampled at 1 kHz and bandpass filtered from 40-5000Hz using custom LabVIEW software.

Study Protocol

Optical and electrical signals were recorded during sinus rhythm. VF was induced by burst pacing the RV at 2-5mA for 60-90s at 10-30Hz. After 1 min of stable VF, perfusion was stopped to induce ischemia. Electrical signals were recorded continuously throughout VF. Optical signals were recorded for 5 seconds at 1-min intervals. All hearts maintained VF for at least 5 minutes.

Data Analysis

Optical and electrical signals were processed and analyzed using custom, Matlab-based analysis software. Optical signals were digitally filtered using a Butterworth low pass filter with a cutoff frequency of 100 Hz. Electrical signals were digitally filtered using a 60 Hz notch filter. Activation time was defined at the maximum first derivative of the voltage signal (dV/dt_{max}). Cycle length (CL) was defined at the time interval between consecutive activations. For optical signals, action potential duration (APD_{60}) was defined at the time interval between activation and 60% repolarization. Diastolic interval (DI) was defined as the time interval between 60% repolarization and the subsequent activation time. AP rise time was defined as the time interval between activation and the AP peak. Calcium transient (CaT) duration (CaD_{60}) was defined as the time between calcium release (maximum of first derivative of calcium signal) to 60% recovery of diastolic calcium levels. Calcium recovery time was defined at the time interval between peak calcium release to 60% recovery. CaT rise time was defined at the time between initial calcium release and peak calcium.

Statistical Analysis

Optical parameters for a single study were determined from a 4 x 4 pixel area in the LV. Transmural activation was compared between the most endocardial and most epicardial electrode pairs on the needle. Summarized data are expressed as the mean of all studies (mean ± SE). Differences in means between VF at each time point and VF onset, and differences between endocardial and epicardial activation were evaluated using a paired Student's t-test. A repeated measures analysis of variance with a Bonferroni correction was performed to test whether VF duration affected CL, APD, DI, CaD, calcium recovery time, and AP and CaT rise time. Results were considered significant at $p<0.05$.

RESULTS

Development of a Transmural Gradient of Activation

A gradient of activation between the endocardium (endo) and epicardium (epi) was found to develop during LDVF in the *in vitro* rabbit, similar to the gradient found in canine [4-7] and human hearts [8]. Figure 1 shows representative electrograms from a single plunge needle in the LV during LDVF, and demonstrates the development of an activation gradient between the endo and epi. From VF onset to 2 min of VF, activations between the endo and epi were mostly 1:1, with similar CLs (Figure 2). As VF progressed, epicardial activation became less regular and slower. By 4 min of VF, epicardial activation was significantly slower than endocardial (111±9 ms vs. 94±8 ms, $p<0.05$) and continued to slow at 5 min while endocardial activation remained the same (139±19 ms vs. 96±5 ms, $p<0.05$). These results are summarized in Figure 2.

Epicardial Action Potentials and Intracellular Calcium Dynamics

Figure 3 shows representative optical APs and CaTs from a single channel during LDVF. In all hearts, APs preceded CaTs throughout VF. During early VF, APs and CaTs were synchronized, with CaTs following every AP. At 4-5 min VF, CaTs became prolonged and less regular, with not every AP always being followed by a CaT, although CaTs never preceded APs. For cases in which a CaT followed an AP, the delay between AP and CaT activation did not significantly change over the course of VF (Figure 4).

As expected from the transmural electrograms, epicardial CL increased significantly from VF onset at 4 min of VF (96±4 ms vs. 80±3 ms, $p<0.05$), and continued to slow at 5 min (112±5 ms, $p<0.05$) (Figure 5). Contrary to other studies [5,9,10], APD_{60} was found to increase slightly at 5 min VF compared to APD_{60} at VF onset (59±3 ms vs. 49±2 ms, $p<0.05$); however, DI increased even more substantially at 4 min (43±3 ms vs. 31±2 ms, $p<0.01$) and 5 min (55±4 ms, $p<0.01$), and the increase in DI accounts for 75% of the increase in CL. The AP rise time also increased slightly at 5 min VF (16±2 ms vs. 12±2 ms, $p<0.05$), although this contribution to increased CL is minimal.

Changes in calcium dynamics appeared to follow a similar time course to electrical changes (Figure 6). CaD_{60} increased significantly from VF onset at 4 minutes VF, and continued to increase at 5 min VF (62±3 ms and 72±4 ms, respectively, vs. 51±3 ms at onset, $p<0.01$). The time from peak calcium to 60% recovery also increased significantly at 4 and 5 min VF (43±4 ms and 50±5 ms, vs. 35±3 ms at onset, $p<0.05$), while the CaT rise time increased at 5 min VF (18±2 ms vs. 15±2 ms, $p<0.05$). The increase in calcium recovery time contributes to ~80% of the increase in CaD_{60}.

DISCUSSION

In this study, we characterized transmural activation and epicardial action potential and intracellular calcium dynamics in an *in vitro* rabbit model of LDVF. The main findings were that: 1) A transmural gradient of activation develops during LDVF in an isolated rabbit heart; 2) epicardial CL increases during LDVF, and the increase in CL is primarily attributed to an increase in DI; 3) CaD increases during LDVF, mostly due to prolonged recovery; and 4) APs always preceded CaTs, although CaTs did not always follow APs during late VF.

This work provides a novel characterization of the *in vitro* rabbit model of LDVF. Many studies investigating VF have assessed electrical or calcium dynamics during short duration VF [12,13], post-defibrillation [14,15], or during LDVF in perfused hearts [16,17]. Few studies have evaluated nonperfused LDVF [5,10], and even fewer have evaluated calcium handling during VF [5]. This study aims to address the lack of studies assessing both electrical and biochemical mechanisms underlying ischemic LDVF.

Transmural Activation Gradient

Previous studies in canine [4-7] and human [8] hearts have established the development of a transmural gradient of activation during LDVF, with epicardial activation decreasing by 2-3 minutes of VF [4-8]; however, no such gradient has yet been documented in the isolated rabbit heart model by transmural assessment. We confirmed the development of a transmural gradient of activation by 4 minutes of VF, with significant slowing of epicardial activation relative to endocardial activation. The later development of the gradient

in the rabbit heart compared to dog and human hearts could be attributed to two things: 1) the location of the needle in the basal LV, since differences in activation rate are heterogeneous; or 2) the first minute of perfused VF may not accurately reflect LDVF *in vivo*. However, the presence of a transmural activation gradient is consistent with prior studies, both *in vivo* and *in vitro*, and our results provide transmural evidence to support its development in rabbits.

Epicardial Action Potentials and Intracellular Calcium Dynamics

Changes in LV epicardial action potentials during LDVF were similar to those found in dog [5], pig [9], and rabbit [10] hearts, with CL increasing primarily as a result of increasing DI. CL and DI increased significantly at 4 minutes of VF, which is consistent with the time course of the development of the transmural activation gradient and slow epicardial activation. Contrary to other studies, which have found a decrease in APD during LDVF [5,9,10], we found a slight increase in APD, along with an increase in the AP rise time. The use of blebbistatin as an electromechanical uncoupler may falsely increase APD, due to its electrophysiological effects in rabbit hearts [13]. Blebbistatin has been found to prolong APD at baseline cycle lengths by ~28% in rabbits; however, the effect of blebbistatin on APD prolongation increases as CL decreases, with increases as high as 50% at a CL of 200ms [13]. The increase in APD in our studies was ~20% by 5 min LDVF, which is well within the range of APD prolongation that may be expected from blebbistatin. Additionally, APD shortening at rapid heart rates has been found to occur as a result of ATP depletion and ATP-sensitive potassium channel (K_{ATP}) activation in mechanically working hearts [19,20]. In hearts that are electromechanically uncoupled with blebbistatin, ATP may be preserved due to the lack of mechanical contraction, which could prevent the degree of K_{ATP} activation

necessary to significantly shorten APD. Furthermore, it should be noted that the increase in APD in these studies contributes a relatively small amount to the overall increasing CL; >75% of the CL increase can be attributed to the increase in DI, which increases by >170% by 5 min of VF. These results are consistent with prior studies in canine and porcine hearts [5,9,10] and suggest that the increase in CL is primarily the result of an increasing DI. A potential cause of the significant increase in DI could be an increase in post-repolarization refractoriness as VF progresses, contributing to a decreased activation rate and increasing epicardial CL. However, this study cannot fully assess the role of post-repolarization refractoriness without evaluating tissue excitability during VF. Robertson et al. [9] found that although the minimum DI increased during LDVF in porcine hearts, the activation rate during VF could be accelerated by electrical stimulation, suggesting that while refractoriness may increase during VF, the increase in DI cannot be attributed to refractoriness alone. They also found that although the tissue was excitable at shorter CLs during VF, wavefront propagation of paced tissue was diminished [9]. This poor propagation could be the result of electrical uncoupling and decreased gap junctional conductance, as is found to occur in ischemia [21] and heart failure [22].

Throughout LDVF, APs always preceded CaTs, with a consistent delay between APs and CaTs. 1:1 synchronization was maintained for the first 3 min of VF. At 4-5 min of VF, synchronization decreased, with not every AP being followed by a distinct CaT. CaTs became increasingly prolonged, with CaD increasing by >140% by 5 min of VF. The increase in CaD was largely due to delayed recovery time to baseline diastolic calcium levels. This delay likely indicates impaired calcium reuptake by the sarcoplasmic reticulum (SR), which has also been found in ischemia [23] and heart failure [24]. Impaired SR calcium

handling has been shown to lead to calcium overload that may result in early afterdepolarizations (EADs), delayed afterdepolarizations (DADs), and triggered activity [25-29], and could contribute to LDVF maintenance and post-defibrillation arrhythmias [26,28,29]. However, triggered activity related to calcium overload did not appear to play a role in the electrical changes that occurred during our studies. We found no evidence of spontaneous calcium release (SCR) preceding APs during 5 min of LDVF, suggesting that APs always triggered CaTs during VF. These results are consistent with studies conducted in canine [5] and porcine hearts [30], which also found no evidence of SCR occurrence during VF. Other studies have shown that calcium overload during VF can lead to failed electrical defibrillation or post-shock refibrillation [28,29], which may be due to SCRs as a result of continued calcium overload after defibrillation [28]. Thus, although triggered activity due to calcium overload and SCRs did not appear to contribute significantly to LDVF maintenance in our studies, it may play a role in VF beyond 5 min or in defibrillation.

The time course of changes in action potential and intracellular calcium dynamics were similar and consistent with the timing of the development of an activation gradient. Although it is likely that altered intracellular calcium dynamics impact electrical activity during LDVF, the concurrent timing of AP and CaT changes, as well as the lack of SCR occurrence, suggests that alterations in intracellular calcium handling do not contribute directly to the initiation of decreased epicardial activation during LDVF.

Limitations

The heart was perfused during the first minute of LDVF, to ensure stable VF had been induced. While this model may not precisely reflect a clinical presentation of ischemic

VF, it is possible patients may have at least partial blood flow during early VF. Transmural recordings were constrained to the basal LV so as not to interfere with the optical mapping field of view. Although our data confirms the development of a transmural gradient of activation rate, the timing of development and degree of difference between endocardial and epicardial activation times may not accurately reflect that of the whole heart. Nonetheless, epicardial action potentials from optical mapping over the LV surface validate the timing of decreased epicardial activation found by transmural recordings. The use of blebbistatin has been shown to prolong APD in isolated rabbit hearts, and may alter electrophysiological characteristics during VF [13]. However, the degree of APD prolongation found in our studies was less than previously documented as a result of blebbistatin use, and is likely not a major issue in our studies.

Acknowledgements

We would like to acknowledge Frank Vance, Sharon Melnick, and Shannon Salter for their technical support.

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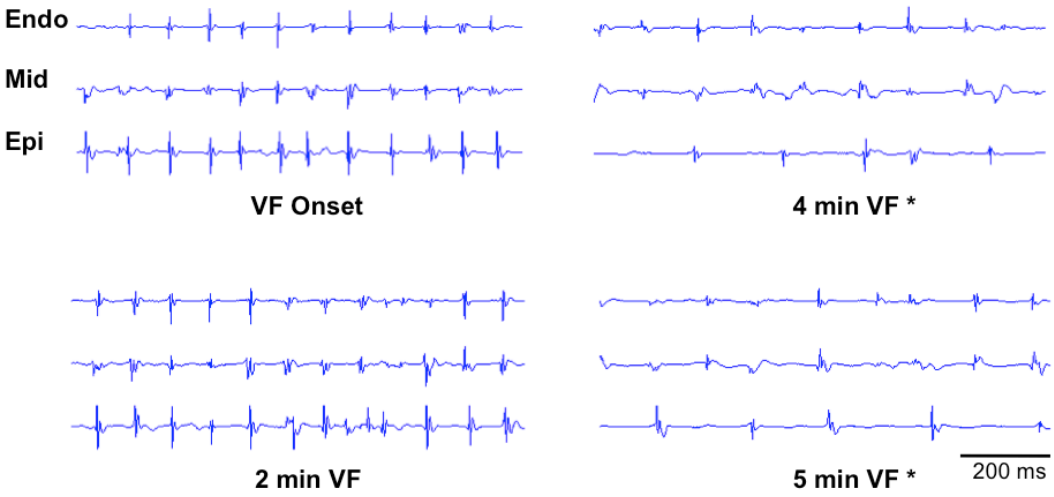

Figure 1: Transmural Electrograms. Representative electrograms from a single plunge needle during LDVF are shown. At each time point, electrograms from the endocardium (Endo), midmyocardium (Mid), and epicardium (Epi) are included. Activations at VF onset and 2 min VF are regular, with similar CLs in the Endo, Mid, and Epi. At 4 min VF, activations are less distinct, and Epi activation is considerably slower than Endo. Endo:Epi activations are no longer 1:1. Epi activation continues to slow at 5 min VF. (* denotes statistical significance between Endo and Epi activation rate, $p<0.05$).

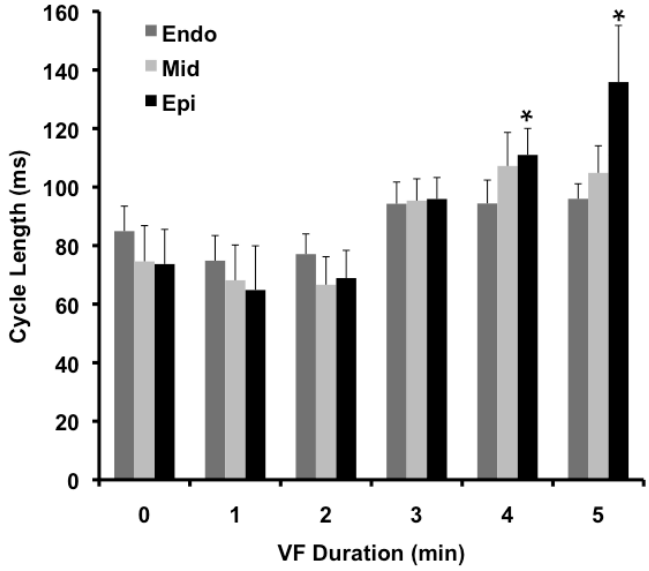

Figure 2: Transmural Activation Gradient during LDVF. Cycle lengths are shown for Endo, Mid, and Epi at each minute of LDVF (n=4). A significant gradient of activation between Endo to Epi developed at 4 min VF, with an increased Epi CL, which continued to increase at 5 min VF ($p<0.05$).

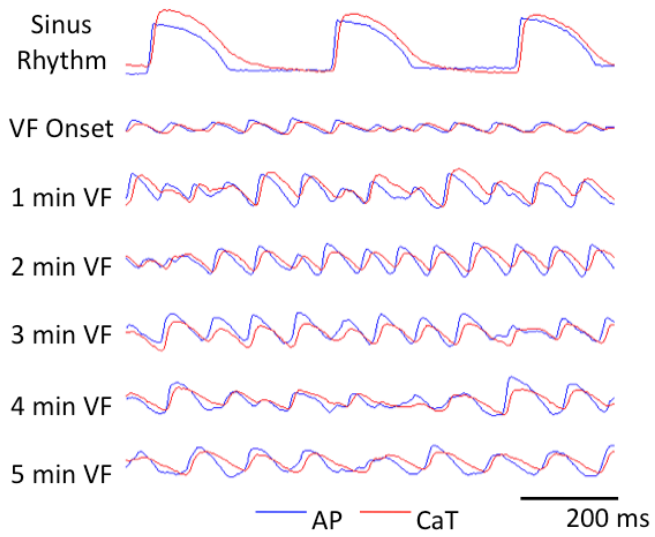

Figure 3: Optical APs and CaTs during LDVF. Representative optical APs and CaTs from a single channel during sinus rhythm and LDVF are shown. Throughout LDVF APs always precede CaTs. During early VF, APs and CaTs are 1:1. At 4-5 minutes of VF, CaTs are noticeably prolonged, and not every AP is followed by a CaT.

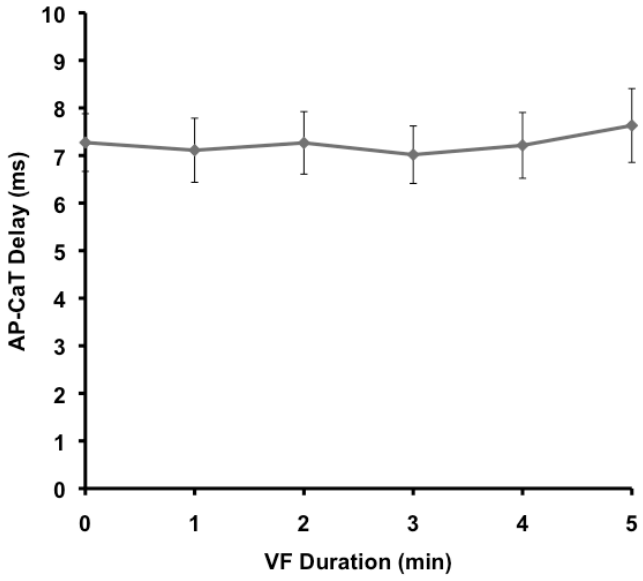

Figure 4: AP-CaT Delay during LDVF. The delay between AP activation and calcium release is summarized at each minute of VF (n=5). No change in the AP-CaT delay was found during 5 minutes of LDVF.

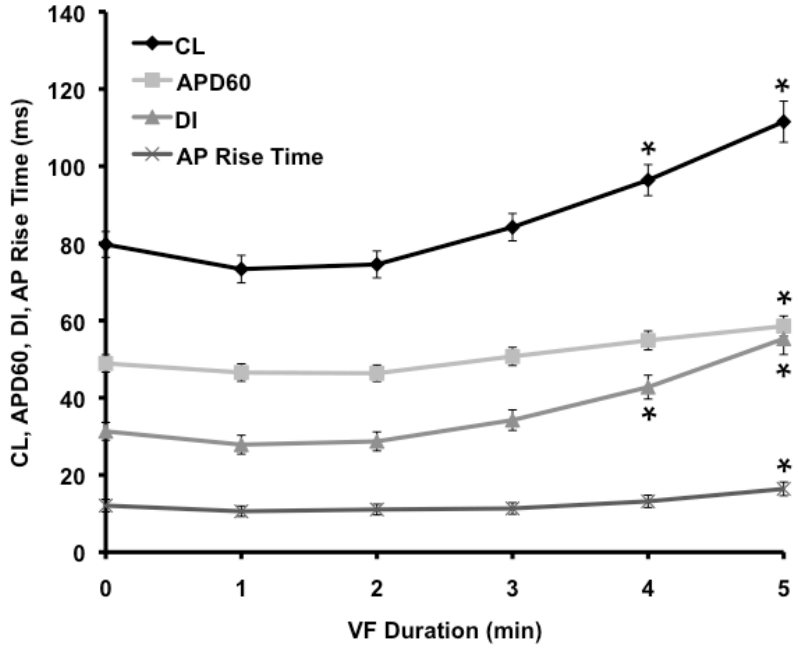

Figure 5: AP Dynamics during LDVF. AP dynamics at each minute of LDVF are summarized (n=5). CL increases significantly at 4 min VF, and continues to increase at 5 min VF. APD_{60} increased slightly at 5 min VF, but the majority (>75%) of the increase in CL is attributed to a significant increase in DI at 4 and 5 min. AP rise time increased slightly at 5 min VF, which may account for the slight increase in APD_{60}. (* denotes statistical significance, $p<0.05$)

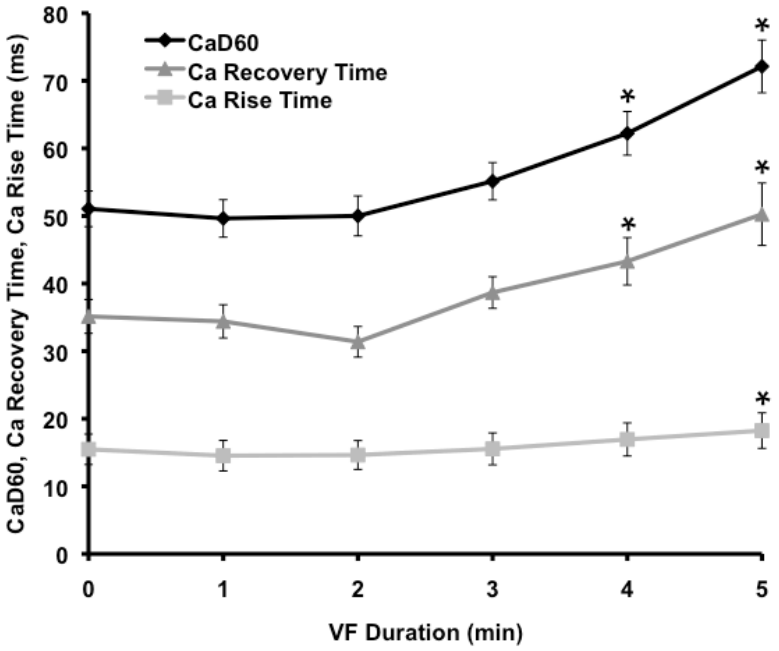

Figure 6: Calcium Dynamics during LDVF. Calcium dynamics at each minute of VF are summarized (n=5). CaD_{60} increases significantly at 4 min of VF, and continues to increase at 5 min. Calcium recovery time also increases significantly at 4 and 5 min of VF, and the delayed return to diastolic calcium levels accounts for most of the increase in CaD. Calcium rise time increases slightly at 5 min VF, but this increase does not contribute greatly to prolonged CaD. (* denotes statistical significance, $p<0.05$)

CONCLUSION

A limited understanding of the electrophysiological and biochemical mechanisms underlying LDVF has hindered improvements in therapeutic treatments to prevent death from cardiac arrest. This study investigated epicardial and transmural activation and intracellular calcium dynamics simultaneously by utilizing an integrated approach that combined optical and electrical mapping in a rabbit model of LDVF that has not been well characterized. Results in the isolated rabbit heart were similar to results found in other animal species, validating the efficacy of the model. A transmural activation gradient developed during LDVF, which was associated with concurrent changes in epicardial action potentials and intracellular calcium. Epicardial activation decreased, while the diastolic interval increased, which could suggest increased refractoriness. CaTs were significantly prolonged and the recovery time to baseline diastolic calcium levels was delayed, indicating impaired calcium reuptake. Despite these changes, APs always preceded CaTs, although some dissociation did occur during late VF, with not every AP being followed by a CaT. These results suggest that although development of the transmural gradient and slow epicardial activation may be associated with changes in intracellular calcium dynamics, impaired calcium handling *per se* does not seem to contribute directly to the decreased epicardial activation. However, impaired calcium handling may still contribute to LDVF maintenance and post-shock arrhythmia, and other biochemical mechanisms may underlie both altered epicardial activation and calcium dynamics during LDVF. Findings from these studies contribute to our knowledge of the

mechanisms underlying prolonged VF, which is fundamental to the development of novel therapies.

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APPENDIX

IACUC APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 16-Jun-2017

TO: Pogwizd, Steven

FROM: Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 16-Jun-2017.

Protocol PI:	Pogwizd, Steven
Title:	Arrhythmia Mechanisms in Heart Failure
Sponsor:	UAB DEPARTMENT
Animal Project Number (APN):	IACUC-08356

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 15-Jun-2020.

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
CH19 Suite 403 | CH19 Suite 403
933 19th Street South | 1530 3rd Ave S
(205) 934-7692 | Birmingham, AL 35294-0019
Fax (205) 934-1188 |

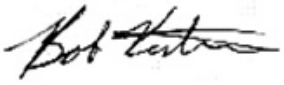
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

Notice of Approval for Protocol Modification

DATE: August 21, 2014

TO: STEVEN POGWIZD, M.D.
VH -B140
(205) 975-4710

FROM: 

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Arrhythmia Mechanisms in Heart Failure
Sponsor: Internal
Animal Project_Number: 140208356

On August 21, 2014, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the modification as described: Personnel: Ashleigh Hood The sponsor for this project may require notification of modification(s) approved by the IACUC but not included in the original grant proposal/experimental plan; please inform the sponsor if necessary.

The following species and numbers of animals reflect this modification.

Species	Use Category	Number In Category
Rabbits	B	Zero - Procedural modification only

The IACUC is required to conduct continuing review of approved studies. This study is scheduled for annual review on of before February 3, 2015. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files.

Refer to Animal Protocol Number (APN) 140208356 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

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