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ADRENERGIC STIMULATION IN ACUTE HYPERGLYCEMIA: EFFECTS ON CELLULAR AND TISSUE LEVEL MURINE CARDIAC ELECTROPHYSIOLOGY

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THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in
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By

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Lexington, Kentucky

Director: Dr. Abhijit Patwardhan, Professor of Biomedical Engineering

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Abstract of Thesis

ADRENERGIC STIMULATION IN ACUTE HYPERGLYCEMIA: EFFECTS ON CELLULAR AND TISSUE LEVEL MURINE CARDIAC ELECTROPHYSIOLOGY

Cardiovascular complications associated with elevated levels of glucose in the blood (Hyperglycemia, HG) is a growing health concern. HG is known to be associated with a variety of cardiovascular morbidities including higher incidence of electrical disturbances. Although effects of chronic HG have been widely investigated, electrophysiological effects of acute hyperglycemia are relatively less known. Further, hyperglycemic effects on adrenergic response is not widely investigated. We used excised ventricular tissues from mice to record trans-membrane potentials during a variety of pacing protocols to investigate cellular/tissue level electrophysiological effects of acute hyperglycemia and adrenergic stimulation (1μM Isoproterenol, a β-adrenergic agonist). A custom program was used to compute action potential durations (APD), maximal rates of depolarization (dv/dtmax), and action potential amplitudes (APA) from the recorded trans-membrane potentials. From these computed measures, electrical restitution and alternans threshold were quantified. Restitution was quantified using the Standard Protocol (SP; basic cycle length BCL= 200ms), Dynamic Protocol (DP; 200-40ms or until blockade) and a novel diastolic interval (DI) control protocol with Sinusoidal Changes in DI. Results from 6 mice show that acute hyperglycemia causes prolongation of the APD. Effects of adrenergic stimulation during acute hyperglycemia were partially blunted compared with non-hyperglycemic state, i.e. hyperglycemia minimized the decrease in APD that was produced by adrenergic stimulation. Similar, but less consistent (across animals) effects were seen in other electrophysiological parameters such as alternans threshold. These results show that acute hyperglycemia may itself alter cellular level electrophysiology of myocytes and importantly, modify adrenergic response. These results suggest that in addition to long term re-modeling that occurs in diabetes, acute changes in glucose levels also affect electrical function and further may contribute to systemically observed changes in diabetes by blunting adrenergic response. Therefore, further investigation into the electrophysiological effects of acute changes in glucose levels are warranted.

Keywords: Action potential duration (APD), acute hyperglycemia, maximal rates of depolarization (dv/dtmax) action potential amplitudes (APA), Dynamic protocol, Standard protocol, Sinusoidal DI control

Sridevi Thyagarajan

January 15, 2018

ADRENERGIC STIMULATION IN ACUTE HYPERGLYCEMIA: EFFECTS ON CELLULAR AND TISSUE LEVEL MURINE CARDIAC ELECTROPHYSIOLOGY

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January 15, 2018

Dedicating this to my parents, elder brother, sister-in-law, in-laws and to my dear husband ANAND IYER, for all their prayers, support and trust.
Thank You

Acknowledgements

I would like to take this opportunity and Dr. Abhijit Patwardhan for his continuous guidance and support throughout my Graduate studies. He has been very supportive, understanding and encouraging.

Table of contents

LIST OF TABLES

LIST OF FIGURES

Chapter 1: Introduction

1.1 Diabetes and its prevalence

Diabetes Mellitus (DM) is the 7th leading cause of death worldwide. The Centers for Disease control and Prevention (CDC) estimate that around 9.3% of world population has DM and that it can increase two-fold by 2050 [8]. In the United States, 10.3 million Americans are diagnosed with DM and 5.4 million Americans are left undiagnosed [10]. Diabetes occurs when the body is not able control the increase in plasma glucose levels. People with diabetes are vulnerable to multiple complex diseases. This complexity includes cardiovascular diseases (CVD) and microvascular diseases. CVD is the most common and the death rate in the United States is 1.7 times more in DM diagnosed patients [53]. CVDs are considered to cause death in about 65% of diabetic patients and hence DM is considered as the major risk factor for CVD [11].

In humans, fasting glucose levels between 110mg/dL to 126 mg/dL are considered normal, whereas more that 126mg/dL is considered diabetic. Chronic DM is due to the improper functioning of the pancreas to secrete insulin and are mainly of two types. Type 1 DM, also known as juvenile diabetes or insulin-dependent DM. Type 1 DM is an auto-immune destruction of the body's β-pancreatic cells producing insulin making it insulin deficient [9]. Type 2 DM occurs when the cells fail to respond to insulin (insulin resistant). This is generally associated with obesity. Diabetics are at increased risk of CVD which includes coronary heart disease, heart failure arrhythmia disorders, and sudden cardiac death (SCD). [32].

In general, it has been reported that diabetic individuals have faster heart rates compared to non-diabetic individuals [28, 38].

1.1. a Diabetes and Arrhythmias

In general, it has been reported that diabetic individuals have faster heart rates compared to non-diabetic individuals [28, 38].There is increasing evidence that diabetes leads to cardiac arrhythmogenesis [37]. Arrhythmias are abnormal rhythms of the heart; arrhythmias of the ventricles can include dangerous ones such as ventricular tachycardia or ventricular fibrillation. Diabetes can cause abnormalities in cardiac action potential features and create electrical disturbances. These electrical disturbances begin within a set of beats and then become spatially variable leading to beat to beat variations in electrophysiological (EP) features of an AP, known as electrical alternans.

One of the common arrhythmogenic mechanism is reentry. Here, an activation front loops back on itself by activating tissue recovering from refractoriness. This looping back can arise due to abnormalities from conduction and repolarization in addition to obstacles such as infarcts. When the conduction velocity is disturbed due to myocardial ischemia or when the repolarization is disturbed by ion channel dysfunction, increased adrenergic activity and calcium overload, these factors mediate arrhythmogenesis in DM patients. Ventricular arrhythmias are thought to underlie SCD in both T1DM and T2DM[29].

1.1 b Electrophysiological (EP)changes due to elevated glucose levels

P. Bass et al showed that chronic hyperglycemia (HG) is associated with prolongation of the action potential duration (APD) of rat ventricular muscles [39] and hence alter the electrical and mechanical function of the heart [38]. Several studies show that APD prolongation is the most prominent EP change observed from diabetic hearts[32, 33]. This prolongation makes the tissue more vulnerable to arrhythmias and SCD.

1.1.c Relevance to ECG signal

The ECG Signal records the electrical activity of the heart and each wave in the ECG corresponds to the different phases of Action Potentials (AP) throughout the heart. The Q-T interval corresponds to the duration of all of the APs from the entire ventricles. QT interval analysis can predict cardiac death in several disease states like chronic heart failure [28]. Chronic hyperglycemia is known to prolong the APD and its corresponding QT interval. This is important to understand because alternans in the QT interval will lead to ventricular arrhythmias.

1.2 Hyperglycemia

Hyperglycemia is considered as one of the major risk factors in cardiovascular diseases [17]. Recent studies show that elevation in glucose levels show an impact on microvascular complications like coronary heart disease [39, 41].

Dario et al. showed in their study that acute increase of plasma glucose levels in normal subjects increased the systolic and diastolic blood pressure, heart rate and plasma catecholamine level, increasing cardiovascular complications [17]. Chronic

hyperglycemia is known to prolong the APD in rat ventricular muscles due to impaired Ca2+ channel [18, 19]. Recently, voltage clamp studies in rat myocytes with induced short-term diabetes (STZ) showed that there was a reduction in the transient outward current (I_{to}) keeping L-type Ca2+ current (I_{Ca}) density unchanged [19,20]. Ionic mechanisms supporting this prolongation of APD in rats with chronic DM (whole cell voltage-clamp techniques) were studied and results show the abnormalities of K+ and Ca2+ currents in chronic diabetic ventricular rat myocytes [21].

Bers et al. reported the modification of Ca2+- Calmodulin dependent protein kinase II (CaMKII) in presence of diabetic hyperglycemia leads to APD prolongation and the onset of cardiac arrhythmias [24].

Effects of acute hyperglycemia at the cellular level have been less reported. Bers et al. performed experiments on isolated rat cardiomyocytes during acute hyperglycemic challenge reporting a decrease in the upstroke velocity and APD prolongation suggesting the involvement of modified CaMKII affecting ionic currents [24]. However, it is still unclear how acute hyperglycemia affects ion channels and modifies CaMKII molecule leading to prolongation of APD. Further, less is understood how acute hyperglycemia can modify the adrenergic response at a tissue level.

1.3 Autonomic nervous system (ANS)

The ANS plays a crucial role in maintenance of homeostasis. The ANS is divided into two: Sympathetic nervous system and Parasympathetic nervous system. It innervates internal organs like the blood vessels and heart. The ANS plays an important role in many systemic diseases such as heart failure or asthma, and the beta-adrenergic

system is often targeted for treatment [12]. The sympathetic nervous system prepares the human body in emergency situations generating the flight or fight response by increasing the heart rate and dilating the airways to allow more inflow of air into the lungs. The parasympathetic system is predominant during quiet conditions or the rest and digest state. Adrenergic receptors, when stimulated, activate the sympathetic nervous system and cause positive tropic effects. Catecholamines like epinephrine, norepinephrine and dopamine generate such responses within the body.

1.4 Adrenergic activity in the cardiovascular system

Adrenergic receptors are activated by the binding of neurotransmitters released from the ANS. The sympathetic (i.e. adrenergic) system exerts three immediate cardiovascular effects by increasing the heart rate (positive chronotropy), increase in the contractile force (positive inotropy) and cardiac relaxation (positive lusitropy). All these effects occur together to generate the 'adrenaline rush' or the flight or fight response. When activated, the sympathetic system releases two catecholamines; norepinephrine (NE, noradrenaline) or epinephrine (E, adrenaline) that binds to the receptors on the heart to bring about the immediate cardiovascular effects.

The adrenergic receptors (ARs) present on the surface of the heart are beta-adrenergic receptors (β-ARs) which are G- protein coupled receptors (stimulating, G_S). β-ARs are of three sub types; β_1, β_2 and β_3 , which in turn are linked to adenylate cyclase, causes a sudden increase in the intracellular cAMP levels causing a rise in the intracellular Ca2+ levels.

The abundance of β receptors present on the myocytes varies by subtype. In general β_1 receptor, represent 75%-80% of the entire adrenergic receptor density, followed by the β_2 AR that comprises of 15%-18% of the entire myocardium and β_3 AR that constitutes the remaining 2%-3%. Responding to catecholamines like NE and E, the β_1 AR's are activated to a higher extent than the β_2 AR.

1.5 Isoproterenol (ISO)

Isoproterenol is a beta-adrenergic agonist that binds to beta adrenergic receptors on the heart, specifically β1 and β2. Once it binds it causes a sudden increase in the intracellular Ca^{2+} ions decreasing APD. Isoproterenol does so by activating adenylate cyclase (AC) increasing the intracellular cyclic adenylic acid (cAMP) which in turn activates protein kinase A (PKA).

Isoproterenol also known as Isoprenaline is a β1 and β2 adrenergic agonist that is used to treat conditions like severe bradycardia (slow heart rate) [41]. Administered intravenously, orally or subcutaneously, this non-selective beta-adrenergic agonist induces positive chronotropic, inotropic and dromotropic (increasing conduction velocity within the heart) effects.

At tissue level, this compound has been shown to have variable changes to the APD in the ventricular myocardium [13]. Bers et al showed an increase in the APD with addition of ISO [23], Giotti et al reported shortening [42] finally Kazuo et al reported there was an increase, decrease and no change in the same study [13]. This shows the inconsistent effects of ISO on the APD of myocytes.

Isoproterenol is known to modify ion currents but to what extent is not well understood. The positive tropic effects by catecholamines like ISO is known to have increased the I_{Ca} [43]. The increase in this inward current is compensated by the increase in the outward currents in the cardiac cells. This compensation ensures the heart comes back to its steady state and not enter a potentially arrhythmic condition. In addition to the ISO-induced increase in Ica there is an activation of the slow delayed rectifier K+ current (IKs), fast delayed rectifier K+ current (IKr) and chloride current (ICl) upon administration of ISO. Variable effects of Isoproterenol (ISO) has been observed in several studies across different animal models [13, 42]. It has been reported that the APD shortened by ISO in models of cats, dogs, humans and rabbits and prolonged APD in pigs and guinea pigs [14].

The aim of this study was to determine whether acute hyperglycemia alters the adrenergic responsiveness of the body at a tissue level and to understand if acute hyperglycemic levels within the body increases or decreases the effectiveness of adrenergic activity.

Chapter 2: Background

2.1 Cardiac Conduction System

The heart is known to beat in a coordinated manner electrically and mechanically. The conduction starts at the Sinoatrial (SA) node (pacemakers of the heart) within the right atrium. These cells control the rhythmic pattern of beats. Once the electrical signals arise from the SA node they travel to the atrioventricular (AV) node setting a delay in activation so that the right atrium empties the blood into the ventricles. From here, the conduction diverges into the left and right sides of the heart through the bundle of His, Purkinje fibers reaching down to the endocardium at the apex of the heart into the ventricular epicardium. This conduction propagates from cell to cell through gap junctions in the intercalated discs that are primarily responsible for the rapid and synchronous depolarization of the myocardium. Myocytes within the heart tissue undergo changes in voltage across their cell membrane that can be measured as a transmembrane potential (TMP). These myocytes undergo depolarization and repolarization during an AP which results in voltage changes in the TMP.

2.2 Transmembrane Potential (TMP) generation

Action potentials vary with shape, duration and ionic currents across different regions of the heart in different animals. These variations correspond to the differences in repolarization stages across different models. Murine AP differs from human cardiac action potential as it lacks the predominant plateau phase. This is because of the rapidly activating potassium currents promoting to faster repolarization stages. The guinea pig AP has an elevated plateau phase but the rat

ventricular AP is relatively short with a 'triangular shape' [36]. Action potential morphology varies in conditions of hyperglycemia.

TMP's are electrical potential differences between the interior and exterior of a cell. These electrical potentials are brief changes in voltage due to the influx and efflux of different ions across the lipid bilayer membrane. When a cell is activated, an AP is generated with a change in its voltage from its resting membrane potential. Movement of ions causes concentration gradients that are then maintained through ion pumps. These ions pumps are membrane proteins that use cellular energy, in the form of ATP, to pump these ions from lower to high concentration.

AP is defined in 4 stages. Phase 0 to Phase 4 is explained using the below figure.

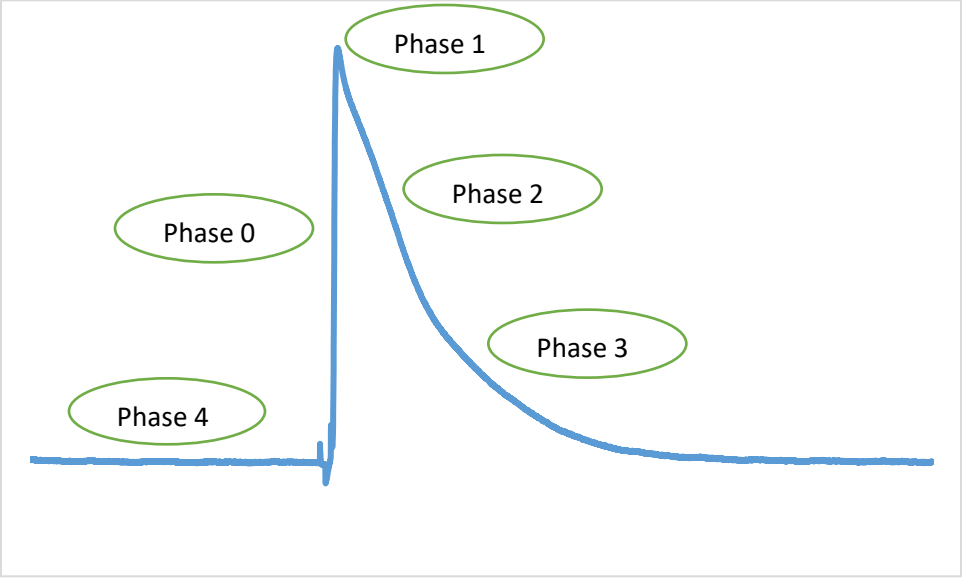


Figure 1: Example of a transmembrane potential showing the 4 stages

Phase 0: Once the cell is stimulated and its membrane crosses the threshold potential, it is able to fire an action potential in an all-or-none manner. This is the depolarization

phase that is due to the influx of Na^+ ions that creates a positive voltage. Na^+ channels are activated causing an increased conductance of Na^+ (g_{Na}) that further open more channels with a rapid influx of Na^+ ions. The TMP changes from a negative to a positive one, but not quite close to the Na^+ equilibrium potential. The rate of depolarization is computed as dv/dt and the maximum rate of depolarization known as the upstroke velocity and is reported as dv/dt_{max} which is the slope of Phase 0.

Phase 1: The 'Notch' is formed in this phase due to changes in two ions. First, there is a rapid inactivation of Na+ channels that eventually reduces the inward flow of Na+ ions and second there is a transient outward current K+ and Cl- known as Ito1 and Ito2 respectively. The Ito1 is the major contributor of the notch.

Phase 2: Plateau phase is formed by the inward Ca^{2+} (I_{Ca2^+}) that allows the movement of Ca^{2+} into the cell and by the outward K^+ ions through the slow delayed rectifier channels (I_{Ks}). The Calcium ions are responsible for the contraction phase of the muscle. This phase is generally not prominent in mouse ventricular myocytes.

Phase 3: The cell rapidly repolarizes by closing the Ca2+ channels and keeping the slow delayed rectifier K+ channels open to allow K+ ions to leak out. This ensures a net positive outward current helping the cell reset to the resting membrane potential. This outward current causes more efflux of K+ ions by activating both rapid delayed rectifier I_{Kr} and inward rectifying I_{K1}.

Phase 4: The cell comes to its resting membrane potential at around -90mV and is contributed by flux of sodium and calcium ions that flow into the cell and potassium and chloride out of the cell.

All these ions with other minor currents and gap junctions between cells help conduct the electrical impulse from one cell to the other. Any changes in the concentration and movement of ions can change the conduction pattern causing irregular, fast (tachycardia) and slow (bradycardia) heart rhythms.

2.3 Electrical Restitution

Electrical restitution explains the relation between the action potential duration (APD) and its preceding Diastolic interval (DI). It is seen that a decrease in preceding DI from beat to beat causes a corresponding decrease in APD and an increase in DI results in increase in APD in the context of electrical restitution, the APD at any DI depends on the previous DI. The presence of alternans can be explained from the slope of the Electrical restitution curve (ERC). The slope of ERC is calculated as ΔAPD/ΔDI (alternans potential) as described by Nohlasco and Dahlen [29]. It is hypothesized that the slope of an APD restitution curve can predict cardiac instability [29] When the slope is greater than 1 the tissue is hypothesized to be susceptible to unstable alternans and activation, i.e. conduction block. Moreover, an activation block can eventually facilitate re-entry leading to cardiac arrhythmias. When the slope is less than 1 the APD disturbances become smaller leading to stable activation. An ERC with slope equal to 1 is at a boundary of stable and unstable behavior and is thought to lead to alternans. Consistently, Franz et al showed that when the slope of ERC is flat or less steep, the tissue is stable and thus antiarrhythmic [45].

Cycle length for a beat is defined as the sum of preceding APD and the following DI CL=DI+APD. Similar to the relationship with DI, as the CL decrease, i.e. inter activation intervals decrease, APD decreases with shortening of CL.

2.4 Alternans

2.4.1 APD Alternans

APD is generally defined as the action potential duration at the 90% level of repolarization (APD=APD90%). When the heart rate increases above a threshold value, the APs for successive beats begin to alternate in an oscillating pattern. This pattern in the form of long-short-long sequences or short-long-short is called APD alternans [6]. Alternans of APD is widely studied at rapid rates of stimulation, i.e. at shorter cycle lengths [3]. Figure 2 shows an example of APD alternans in a mouse ventricular tissue.

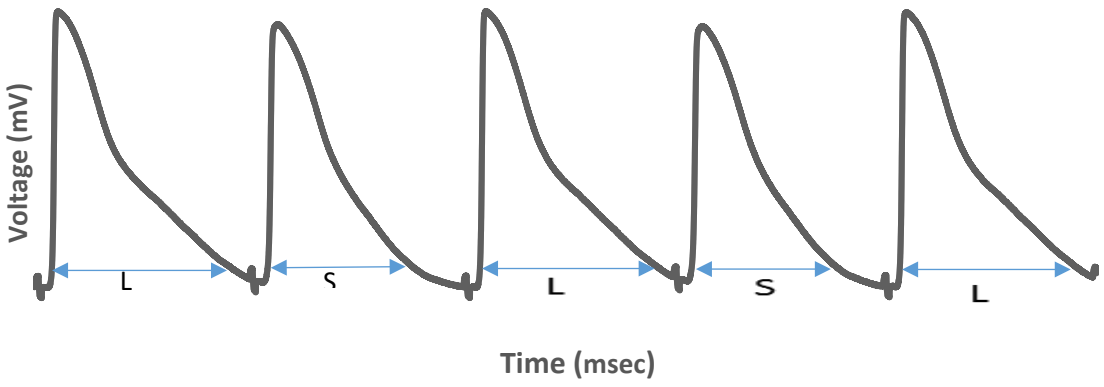


Figure 2: Example of action potential duration (APD) alternans

2.4.2 Concordant and Discordant Alternans

When pacing the tissue fast, it is observed that alternans of APD can vary spatially[1]. Concordant alternans are similar pattern of alternans, long-short-long or short-long-short at different regions of the tissue. Alternatively, alternans will not exhibit a unique pattern, i.e. an area of the tissue alternates as long-short- long and another area alternates as short-long-short sequences, this behavior is called discordant alternans. The transition of concordant to discordant alternans can make the tissue susceptible to ventricular fibrillation[2]. Determination of concordance or discordance requires multiple measurements of APs. While micro-electrode based measurements, such as those in the present study are the gold standard in measurement of TMPs, they are limited in terms of numbers of simultaneous measurements, therefore are not widely used for determination of concord or discordant alternans.

2.4.3 dV/dt_{max} Alternans

Upstroke velocity or the maximum rate of depolarization (dV/dt_{max}) affects conduction and action potential amplitudes (APA) and is a result of sodium channel activation. When a stimulus is available to the cell, of sufficient strength to produce depolarizaton, there is an opening of Na+ channels that allows a rapid entry of sodium ions which then causes further opening of many more Na+ ion channels making the interior of the cell more positive. This sudden increase in positive ions causes the upstroke and the velocity at which this occurs is the upstroke velocity or maximum rate of depolarization. The dV/dt_{max} can also show alternans. When dV/dt_{max} and APD alternates in a similar fashion from beat to beat, such as when the dV/dt_{max} is large

with its subsequent APD being longer, then followed by a lower dvdtmax and a shorter APD they are then considered as in-phase alternans. Similarly, beat to beat changes of dV/dt_{max} and APD when alternate in opposite manner, they are considered as out-of-phase alternans [44]. Out-of-phase alternans are hypothesized to have a stabilizing effect on conduction, however, the out of phase alternans are less common than in phase [44].

2.5 Cardiac memory

Action potential duration (APD) is known to be a function of its preceding diastolic interval (DI) or the previous activation history [6]. An abrupt change in CL or activation rate causes a subsequent change in the APD and is known as an adaptive mechanism to preserve the diastole [49]. From studies, it is seen that continuous changes to the activation rate also results to accumulative and dissipative changes in APD [49]. These changes are based on the previous activation history or previous changes in APD. This phenomenon is called as the memory.

Novel pacing protocols, developed in our laboratory [6] allow explicit control of DI for quantification of memory in cardiac tissues. One of the pacing protocols uses sinusoidal changes in DI where 2 cycles of continuous changes in DI in a smooth fashion (sinusoidal) are produced and corresponding APD's measured. Results show that when stimulating the tissue in a sinusoidal oscillatory manner each value of DI produces two values of APD depending on whether DIs are increasing or decreasing.

This hysteresis obtained observed by the use of this pacing scheme allows us to quantify cardiac memory. It is theorized that memory can suppress alternans and

studies show that memory is known to flatten the restitution curve restoring stable activation of the tissue moving it away from an arrhythmic stage [47,48] .Fox et al. showed that rapid delayed rectifier potassium current (IKr) plays a role in increasing or decreasing the memory effects [16] .

2.6 Ca2+-Calmodulin dependent protein kinase II (CaMKII)

CaMKII is a specific protein kinase that is abundantly expressed on the heart [s] and regulates physiological processes within the heart. It is regulated by the Ca^{2+}/Calmodulin complex (Ca/CaM) and serves in Ca^{2+} homeostasis and Ca^{2+} reuptake within the heart. CaMKII have their regulatory and catalytic subunits that remain intact and auto inhibited. This auto inhibition is disrupted when Ca^{2+}/Calmodulin complex bind to the regulatory domain displacing the auto inhibitory domain that then undergoes phosphorylation making it active. An increase in the intracellular Ca2+ levels during systolic function of the heart leads to an activation and remains active when the Ca2+ levels decline during diastole memorizing the event. Diabetic HG is shown to activate this protein kinase through a modification of the O-linked N-acetyl glucosamine (O-GlcNAc) creating an environmental stress leading to cardiac arrhythmias. Bers at al., reported that elevated levels of glucose corresponding to borderline or severe diabetes (240-500 mg/dL) autonomically activated the CaMKII molecule in rat models. This activation is due to the elevated post-translational modification by O-GlcNAc caused by hyperglycemia contributing to diabetic cardiomyopathy [27]. Hyperglycemic induced CaMKII activation enhances the cardiac SR Ca2+ release increasing Ca2+ spark and

wave frequency leading to arrhythmogenesis. Increased release of Ca can cause an increase in APD via the forward action of the sodium/calcium exchanger. This explain that, when extracellular levels of glucose are acutely elevated, modifications and altered Ca2+ handling makes the tissue susceptible to arrhythmias [27].

Chapter 3: Methods and Analysis

3.1 Experimental setup

3.1. a Animal Preparation

All mice experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky. 6 female mice, strain C57Bl/6 were used for the study with an average weight of 22g - 31g, between the ages of 3- 7 months. Good data were recorded from animals weighing around 28g. These mice were first anaesthetized using Sodium Pentobarbital (150mg/kg, IP) to a deep surgical plane of anesthesia. The heart was then excised from the thoracic cavity. The atria portion of the tissue was carefully removed to avoid stimulations from the sinoatrial node (SA node), then the apex portion was snipped off and the ventricles cut through the septum to expose the endocardial surface of the two ventricles. The tissue, with endocardial side facing up, was immediately put into a 20 x 10 x 5mm Plexiglass tissue chamber and the tissue was superfused with warm modified Tyrode's solution and left to equilibrate for about 30 mins while being paced at 200 ms CL. Biphasic pacing stimuli were delivered through bi-polar platinum-iridium electrodes.

For the result sections below, different number of animals were chosen. For example, Control and HG has n=4 animals tested for CL, out of which n=3 animals were tested for Sin DI. Between Control and ISO n=3 animals were tested for both Sin DI and CL. For the third group, HG + ISO and ISO, n=5 animals were tested for CL and from this n=4 animals were tested for Sin DI. This difference between the numbers of animals

tested between pacing protocols within a group is due to the lack of tissue viability over the period of time during an experiment. Tissue from an animal that was tested for CL would have not viable long enough to be tested for the same buffer using Sin DI protocols. Acquiring data from equal number of animals has been challenging between comparisons of buffers. Bi-phasic pacing stimuli were delivered using platinum-iridium electrodes so that we could observe the dynamics of the AP production under conditions that are hypothesized to inform us about electrical stability/ instability that may affect arrhythmia.

3.1. b Buffer Solutions

Modified Tyrode's solution had composition: 112 mmol/L NaCl, 1 mmol/L KH_2 PO_4, 1.2 mmol/L MgSO4, 5 mmol/L KCl, 50 mmol/L Dextrose and 1.5 mmol/L $CaCl_2$. $NaHCO_3$ was added to the solution to obtain a pH of 7. 3±0.5 (typically 25 mmol/L). The solution was continuously aerated using 95% oxygen and 5% carbon dioxide Furthermore, a constant temperature of 37.0 ±0.5°C was maintained and was checked every 2 hours during the experiment.

For the study, two 1liter buffer solutions were initially made: Control, which was the original Tyrode's solution, to which1 μM Isoproterenol Hydrochloride (ISO) was added to study adrenergic effects in normal conditions. The second liter had high glucose (HG, 3x glucose; 300 mg/dL), as with control buffer, 1 μM Isoproterenol Hydrochloride (ISO) was added to this buffer when investigating ISO+HG condition. Concentration, 1 μM ISO was chosen based on previous study on cardiac AP [52]. In total, 4 different buffers were prepared: Control, ISO, HG and HG + ISO.

3.1. c Electrophysiological recordings

Tissues were stimulated using a 3ms (1.5/1.5) bi-phasic pulse, with 3 times the diastolic threshold at a basic cycle length (BCL) of 200ms. Once the tissue was in the buffer solution, the tissue was paced to equilibrate for about 30 mins before starting the recordings.

Transmembrane potentials (TMP) were recorded from the endocardial side away for the stimulating site. Glass microelectrodes with 10-40MΩ resistance filled with 3M KCl were used to record TMP. Recordings were made only after capture (1:1- for every stimulus, an action potential was present) was obtained. The TMP's were digitized using a commercial data acquisition system (WINDAQ) and simultaneously into a second system where a custom-made DI control program analyzed the TMP in real time to then perform DI control protocols. Details of the real-time control of DI have been reported previously.[4]

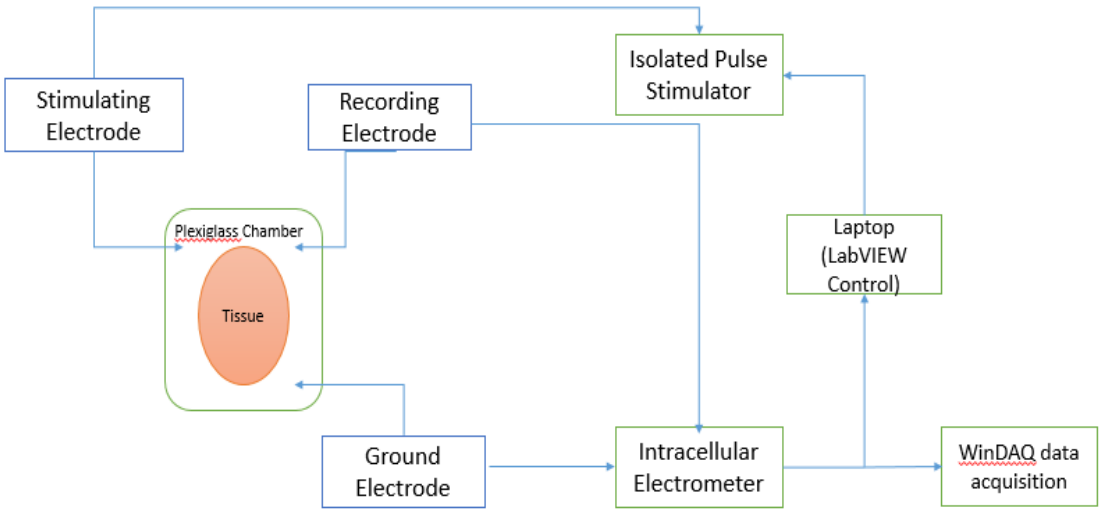


Figure 3: Experimental Setup

3.1. d Data Analysis methods

All digitized TMPs were analyzed offline using custom written programs in MATLAB. The data were low-pass filtered with a cut off frequency of 1000Hz and then digitized at a rate of 30,000 samples/sec. Fiducial points like APmin (starting of an action potential), action potential amplitude (APA) and APD at different levels of repolarization APD_{30}, APD_{50}, APD_{70} and APD_{90} were calculated. In this project action potential duration (APD) is defined as the duration at 90% repolarization level (APD90). All AP's were manually verified for accuracy of detection of each fiduciary point.

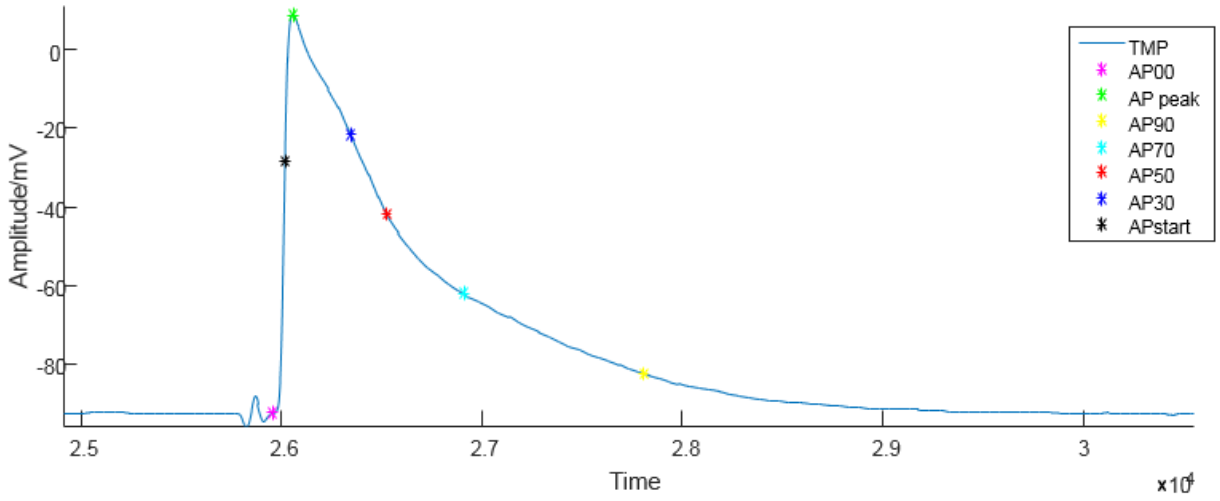


Figure 4: Electrophysiological features recorded from an action potential

3.1 e Alternans

In this study alternans criteria was based on previous studies [51]. Two different changes in successive beats were analyzed to consider them as alternans, an APD ≥ 4ms for at least 5 consecutive AP's and these beats have alternating signs. This was

used classify them as alternans in this study and in previous studies conducted in our lab [44, 46].

3.1. f Chemicals

Isoproterenol (ISO): After switching the buffer from Control to ISO / HG or HG+ISO, the tissue was allowed to acclimatize for 2-3 minutes (pacing at BCL=200msec) and then TMPs were recorded.

3.2 Experimental protocols

3.2.1 Cycle length (CL) Pacing

Restitution kinetics is studied to understand cardiac instability. Conventional methods like the CL pacing protocols predicts the APD behavior to diastolic interval. In CL pacing the stimuli are delivered at a preset interval. For every stimulus an AP is produced and this defines the 1:1 nature. The interval from when this action potential ends to the start of the next action potential becomes the DI for the next AP. Therefore, one does not independently control DI nor APD.

3.2.1a Standard Restitution protocol (SP)

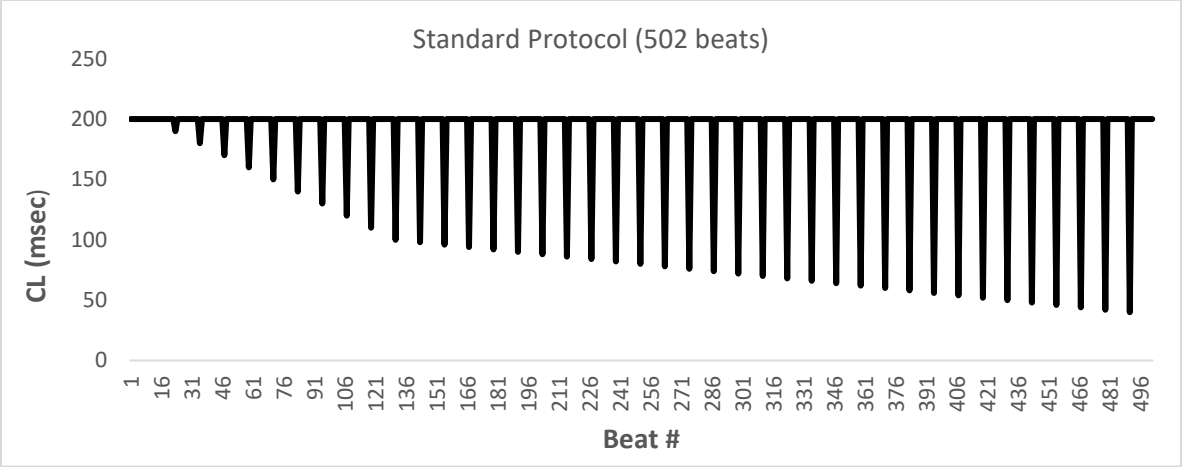


Figure 5: Standard pacing protocol with BCL = 200msec, total duration = 502 beats

Restitution kinetics is studied by understanding the relation of a DI and its dependent APD. One of the widely used protocols to investigate restitution is the standard, i.e. the S1-S2 protocol [50]. In this protocol, a series of 10 pacing pulses (S1) with BCL=200ms are delivered with every 11th pulse (S2) being delivered at different intervals from the previous and last S1 pulse. The S1-S2 pulse interval decrements by 10 ms from 200 ms to 100 ms and then decrements by 2ms from 100 ms to 40 ms or until activation block occurred. The Basic cycle length (BCL) is chosen as 200ms because no alternation in APD occurred at this cycle length. The restitution curve is then plotted between the DI preceding the response (APD) to S2 stimuli and the APD of the S2 stimuli. The motive of studying SP is to understand how the APD changes after an abrupt shortening of the cycle length [50]. The standard protocol investigates the dynamics during sudden changes with minimal adaptation. It explores how AP changes when a change occurs at the same steady state but at different coupling intervals. That is why for standard protocol one paces for N number of beats at the same CL before giving the N+1st stimulus at varying CL.

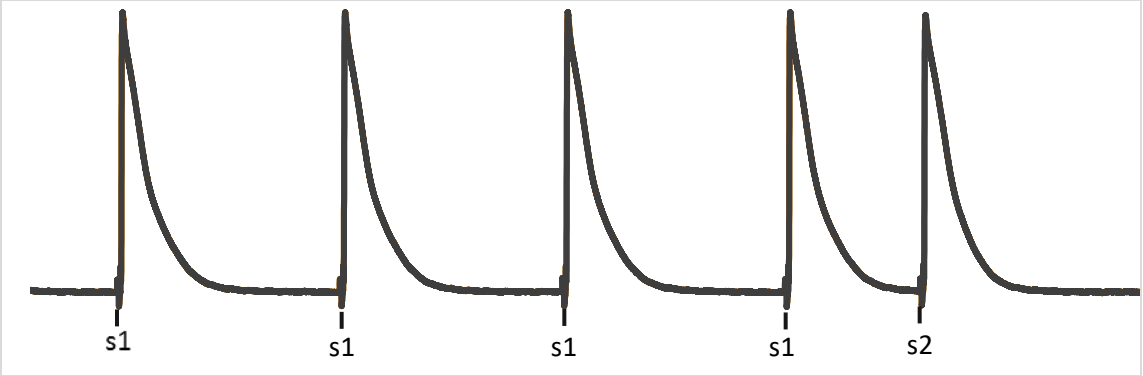


Figure 6: Transmembrane potential recordings during SP

3.2.1b Dynamic Restitution protocol (DP)

The dynamic protocol studies the effects when the pacing rate changes in a continual fashion and includes effect of adaptation, it explores how does AP change when the system has been paced at different rates for a number of beats, i.e. it does not allow the system to go to the same steady state, instead, says that if the system starts operating at different rates, what does the AP change at the end of each of those different states.

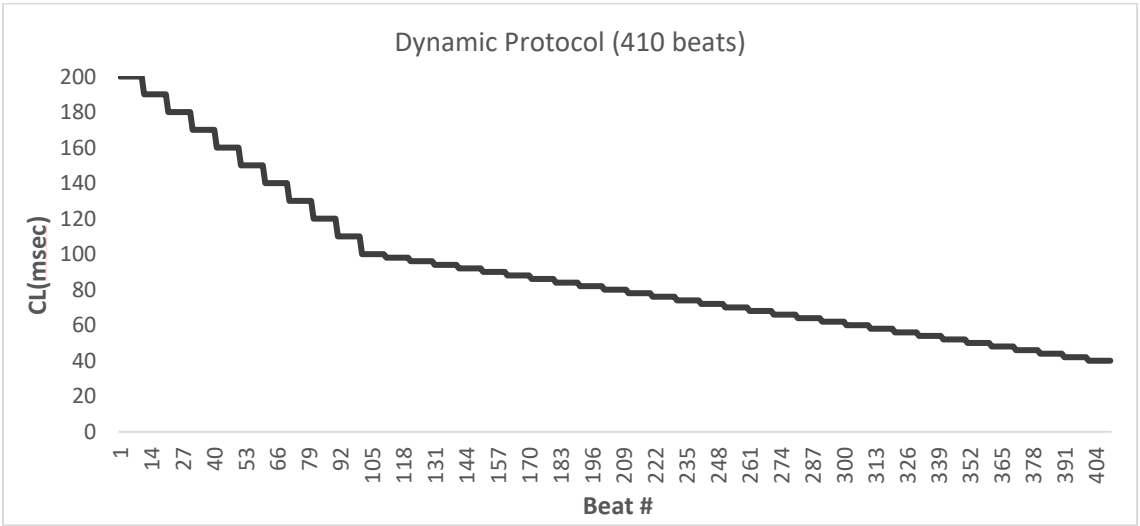


Figure 7: Dynamic pacing protocol with BCL = 200msec, period = 410 beats

The dynamic protocol [50], also known as S1-S1 is where the tissue is paced with a series of 10 pulses starting from a BCL, in our case of 200ms decrementing to 100ms by 10 msec and by 2msec from 100 ms to 40 ms or until block occurs which is when a stimulus fails to elicit an action potential. DP is a step function so that the cell adapts at every step. From this adaptation we study two things, firstly, at what CL does the tissue produce a block or is not able to produce an AP for that specified DI. Secondly, it allows for determination of alternans threshold or at what CL the difference between the consecutive S1 stimuli's APD is greater than 4ms for 5 or more successive beats.

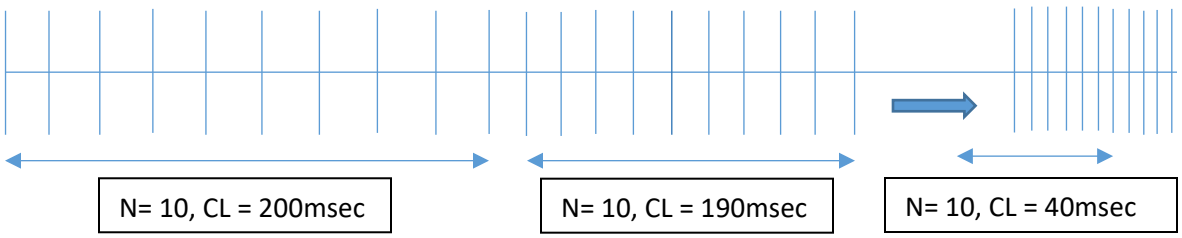


Figure 8: Dynamic pacing protocol sequence with BCL = 200msec, total duration = 410 beats.

3.2.2 Sinusoidal Protocols

To understand characteristic features of hysteresis of restitution and to quantify cardiac memory, we paced the tissue using an oscillatory sequence of DI. Here, we control the independent variable, DI, to study the behavior of the dependent variable, APD (APD= fDI). Moreover, we change DI sequentially and independently from APD to better understand restitution kinetics. This protocol was explained in detail in Wu

and Patwardhan [4]. A smooth function such as a sinusoidal wave is chosen to drive the DI's or activation rate continuously over time to understand how APD's would change. In DI pacing, the pacing stimuli are given such that a preset DI is produced. Therefore in DI pacing DI is independently controlled. The tissue is paced using a feedback control system where APD_{90} of the beat is detected in real time and once the APD_{90} was reached, a stimulus was triggered after a predetermined period which was the desired DI for the successive AP. This explicit DI control have 4 pacing controls all with a mean DI =100ms with a ± 80ms, ±70ms, ±60ms and ±50ms sinusoidal change around its mean. The total duration for Sin ± 80ms and ±70ms is 240 beats and for Sin ±60ms and ±50ms is 220 beats. Each of these Sin DI protocols have two periods, each 100 beats. The tissue was paced for two complete cycles.

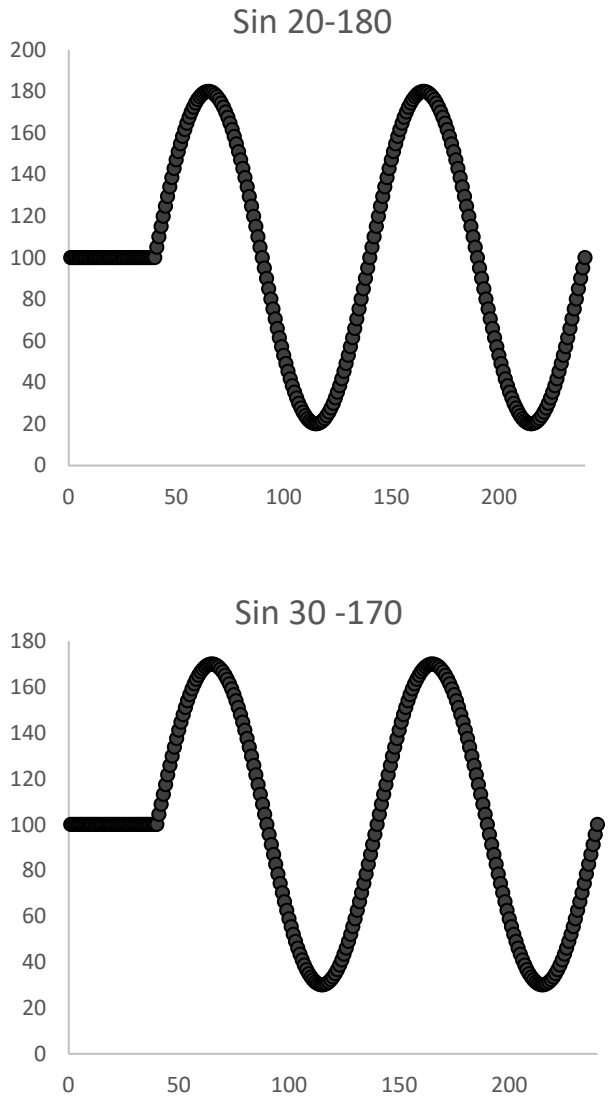
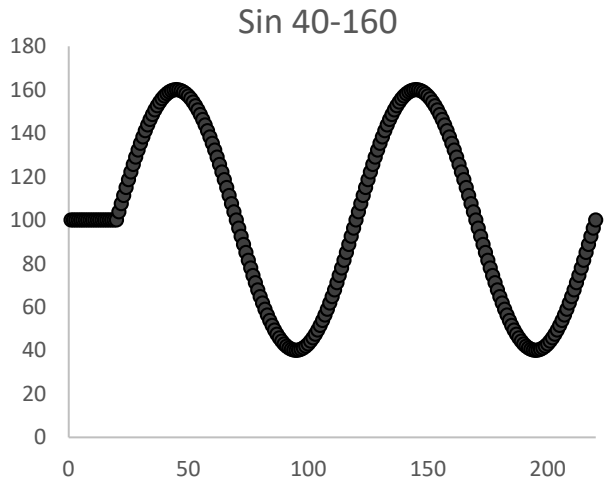


Figure 9 a.) (Top) Sin DI pacing protocol with center DI = 100 msec and range = ±80msec, total duration= 240 beats; b.) (Bottom) Sin DI pacing protocol with center DI = 100 msec and range = ±70msec, total duration = 240 beats



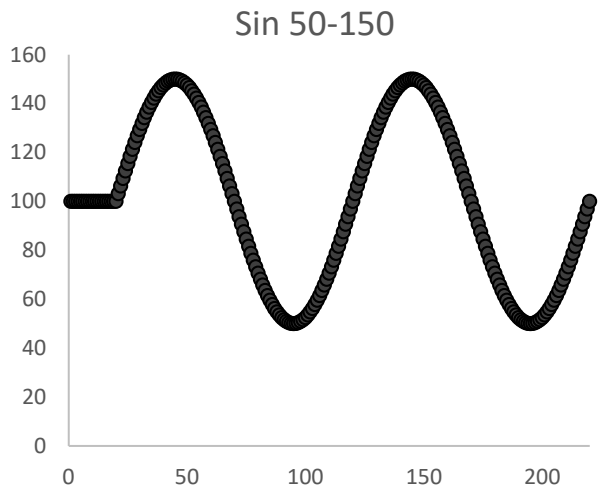


Figure 9 c.) (Top) Sin DI pacing protocol with center DI = 100 msec and range = ±60msec, total duration = 220 beats; d.) (Bottom) Sin DI pacing protocol with center DI = 100 msec and range = ±50msec, total duration = 220 beats.

3.2.3 Constant DI

To study the occurrence of alternans, the tissue was stimulated with 3 sets of Constant DI protocols. The constant DI values were 20, 40 and 60 msec (DI20, DI40 and DI60). In this protocol, the tissue was paced with a series of 200 pulses and a

feedback controller was used such that each AP was preceded by a constant DI as mentioned above to study alternans. The difference between successive DIs (for beat n+1 – for beat n) should be less than 4 msec. In every experiment, the tissue was subjected to these protocols and was seen that most of the data sets could not be used because the DI control was not good.

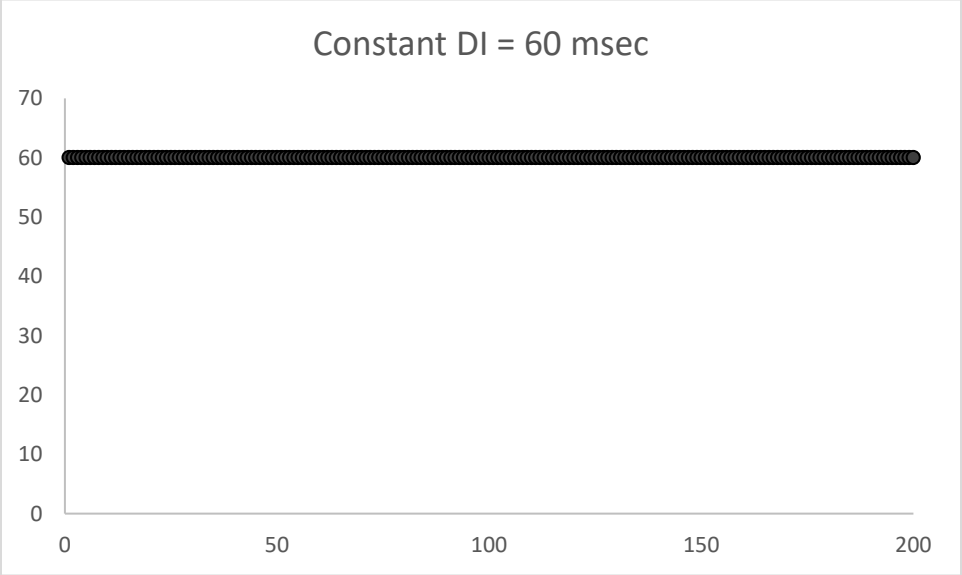


Figure 10: Depiction of constant DI = 60msec, period = 200 beats

Chapter 4: Results and Discussions

Part 1

The first objective of this project was to understand how acute HG by itself could affect action potential characteristics like APD, APA and dvdtmax. These measures were compared with those obtained at control (baseline) conditions. As stated in the methods section, control buffer contained 90mg/dL of glucose and acute HG contained 300 mg/dL of glucose, which is 3 times the glucose in control buffer.

4.1 AP characteristics between Control & HG

An example of TMP recorded at CL=200msec from an animal whose tissue was superfused with Control and HG buffer solutions is shown below (figure 11.a). Average APD's were quantified using different protocols like Sinusoidal DI (n=3), Dynamic Protocol (n=3), Standard protocol (n=4) and constant DI (n=3) animals.

Figure 11.a and 11.b both show APD prolongation in presence of HG compared to Control. But, APA varies in the two, in the first figure the amplitude (APA) had increased in presence of HG by 33% and decreased in the other by 3.2%. It was observed that APA increased across all protocols in 2 of 4 animals and decreased in the other two.

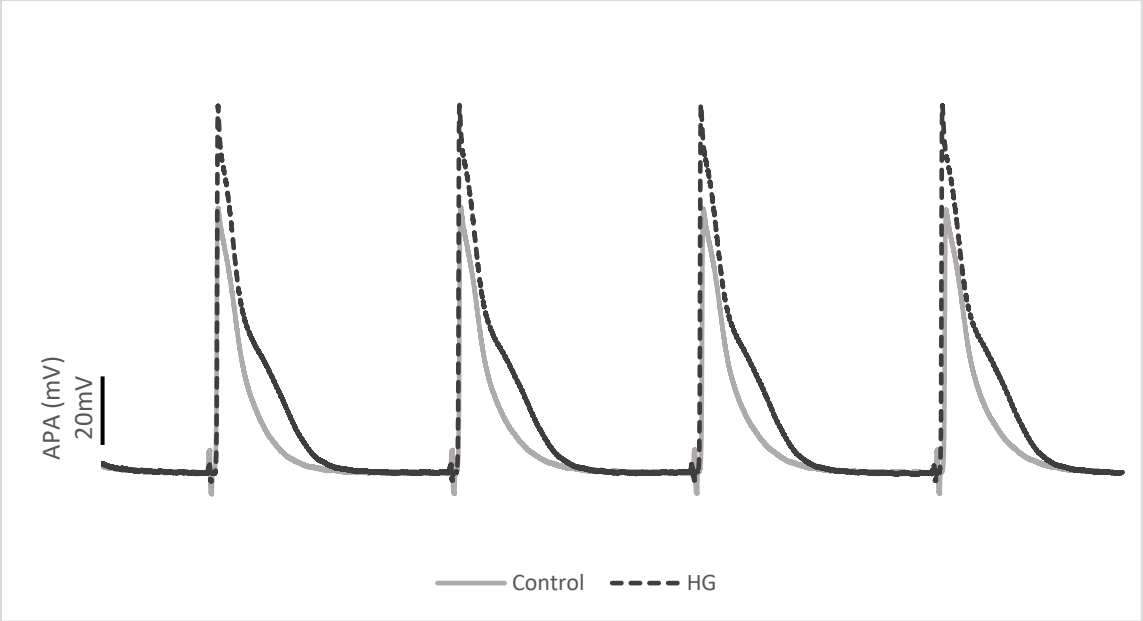


Figure 11.a: Action potentials showing APD prolongation along with an increase in APA in presence of HG compared to Control at CL= 200ms. APD prolongation was at an average of 20% in presence of HG.

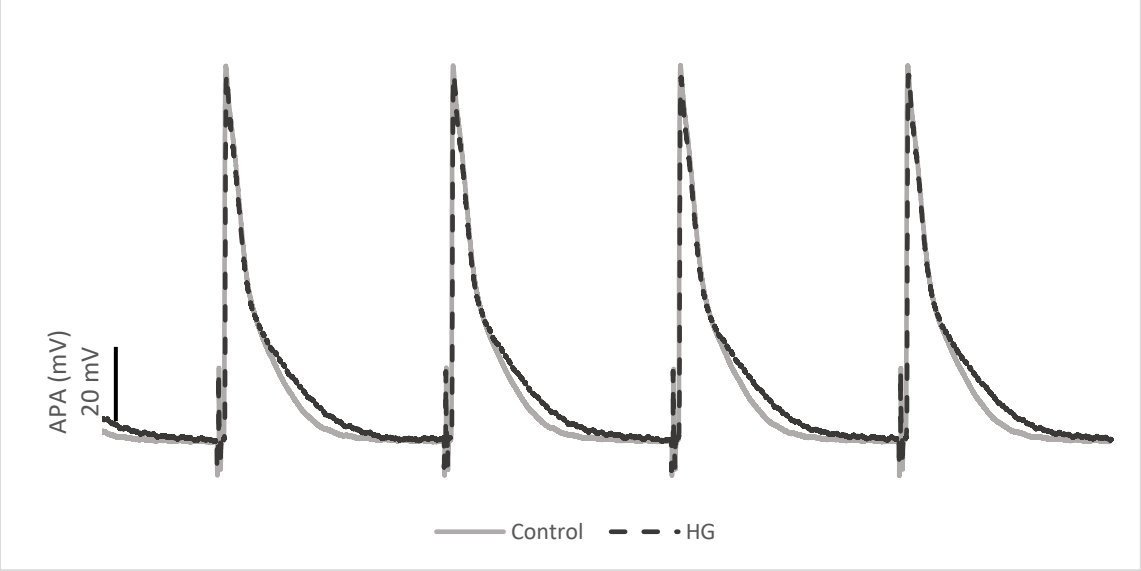


Figure 11.b: Action potentials showing APD prolongation along with a decrease in APA in presence of HG compared to Control at CL= 200ms. APD prolongation was at an average of 19% in presence of HG.

4.2 Effect of HG on APD

Data from 4 tissues which were paced at different activation rates (Cycle length protocol) and explicit DI control were used to determine the effect of HG on APD.

4.2 a Effect of HG on APD compared to Control using CL- dependent changes

To study the effect of HG on APD, the tissues were paced at different activation rates as explained for Standard (n=4) and Dynamic protocols (n=3). An overall increase in APD was observed across the two protocols in the presence of HG compared with Control. Figures (12.a and 12.b) are examples of the restitutions curves (Dynamic & Standard) showing APD prolongation for the same CL in presence of HG.

It is known that as CL decreases, APD also decreases and a block is produced at a CL that produces no AP for that stimuli. Figure 12.a is an example, from one tissue of Dynamic restitution curve (S1-S1) where APD from the last S1 beat is plotted against its preceding DI from the same pulse train. From the 3 animals tested with dynamic protocol, 2 animals showed a prolongation and 1 animal showed an opposite effect.

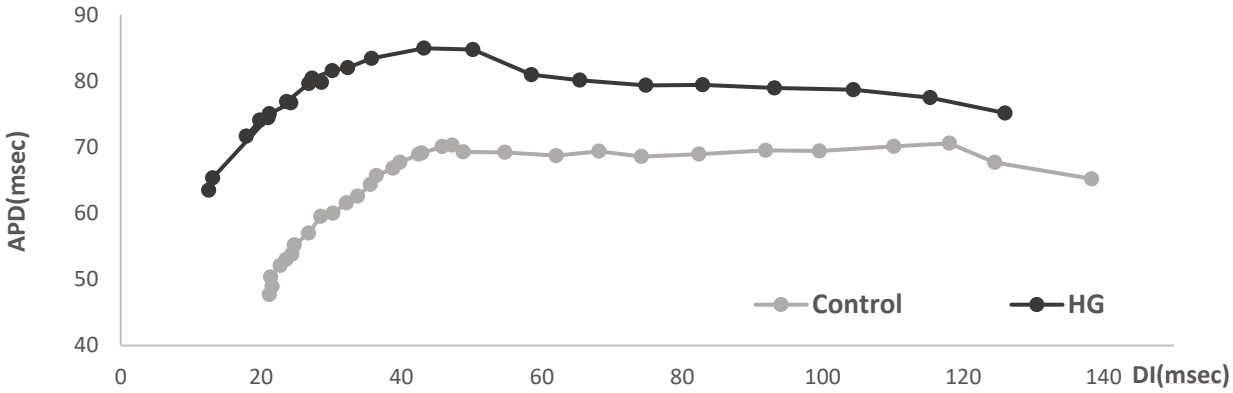


Figure 12.a: Dynamic restitution curve (S1-S1) showing APD prolongations in presence of HG compared to Control. Data are from one tissue.

Figure 12.b shows an example (from one tissue) of standard restitution curve (S1-S2) when APD of the S2 beat was plotted against DI of the preceding S1 beat. From the 4 animals tested with the SP 3 of 4 animals showed APD prolongation in presence of HG, an opposite effect was seen in 1 animal.

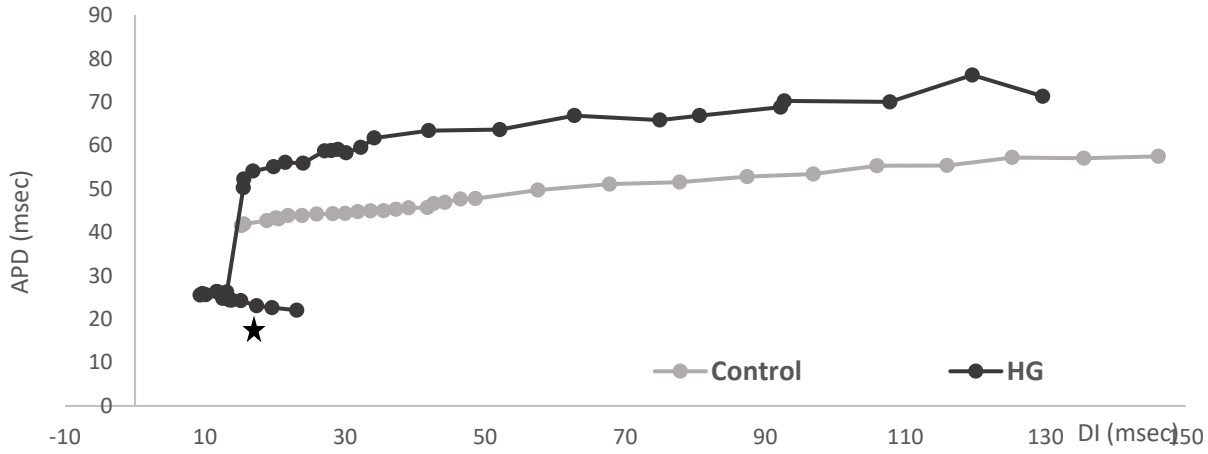


Figure 12.b: Standard restitution curve (S1-S2) showing APD prolongations in presence of HG compared to Control. Example is from one mouse.

Few animals showed adaptation, which is slow and monotonic change in APD as the average rate of activation increases, this can be seen in the figure 12.2 (indicated as a 'star'). Here, as the CL decreases, APD decreases but with an increase in DI, decreasing the alternans potential. This feature is observed in animals tested for CL dependent changes.

In the below tables, are values of APD90 (msec), dvdtmax (mV/msec) and APA (mV) from 3 animals (DP) for three different cycle lengths 200msec, 160msec and 120 msec. While APD90 (msec) values has increased for these different CL's in all the 3 animals, their corresponding APA (mV) and dvdtmax (mV/msec) showed

inconsistent results. The table (n=3) shows that the increase in APD90 ranged 4% to 17% for CL = 200msec, 13% to 18% for CL = 160 msec and 11% to 19% for CL =120 msec. These results also show that as the pacing rate increased, i.e. CL decreased, APD90 was longer (green upward arrow) in presence of HG compared to Control.

Table 1: Average values of APD 90 durations (msec), APA (mV) and dvdtmax (mV/msec) taken from 10 beats paced with CL= 200msec from Dynamic protocol (n=3) showing average increase in all 3 animals for APD90 durations (msec); decrease in 2 animals and increase in 1 animal for APA (mV); and increase in 2 animals and decrease in 1 animal.

Mouse #	Study	Durations	APA	dv/dt (max)
		APD90	(mV)	(mV/ms)
1	C	62.647	83.723	42.139
	HG	65.023	82.154	29.172
	%	3.65 ↑	-1.9	-44.45
4	C	65.173	98.204	48.044
	HG	77.19	95.194	49.746
	%	15.56 ↑	-3.16	3.42 ↑
5	C	63.537	71.662	24.969
	HG	76.427	97.356	47.45
	%	16.86 ↑	23.39 ↑	47.37 ↑
average	%	12.0233333	6.11	2.113333

Table 2: Average values of APD 90 durations (msec), APA (mV) and dvdtmax (mV/msec) taken from 10 beats paced with CL= 160msec from Dynamic protocol (n=3) showing average increase in all 3 animals for APD90 durations (msec), decrease in 2 animals and increase in 1 animal for APA (mV) and increase in 2 animals and decrease in 1 animal for dvdtmax (mV/msec).

Mouse #	Study	Durations APD90	APA (mV)	dv/dt (max) (mV/ms)
1	C	61.293	85.801	43.432
	HG	71.583	85.622	26.42
	%	14.37 ↑	-0.2	-64.39
4	C	72.283	97.56	44.45
	HG	88.403	93.59	46.32
	%	18.23 ↑	-4.24	4.037 ↑
5	C	68.37	65.207	19.449
	HG	78.793	95.373	44.772
	%	13.22 ↑	31.67 ↑	56.55 ↑
average	%	15.27	9.076	-1.27

Table 3: Average values of APD 90 durations (msec), APA (mV) and dvdtmax (mV/msec) taken from 10 beats paced with CL= 120msec from Dynamic protocol (n=3) showing average increase in all 3 animals for APD90 durations (msec), decrease in 2 animals and increase in 1 animal for APA (mV) and increase in 2 animals and decrease in 1 animal for dvdtmax (mV/msec).

Mouse #	Study	Durations APD90	APA (mV)	dv/dt (max) (mV/ms)
1	C	54.477	84.07	44.63
	HG	67.047	84.96	21.99
	%	18.78 ↑	1.04 ↑	-102.9
4	C	70.64	96.16	41.75
	HG	80.882	89.98	41.31
	%	11.38 ↑	-14.9	-1.065
5	C	62.867	59.812	14.539
	HG	76.74	92.517	40.859
	%	18.077 ↑	35.33 ↑	64.41 ↑
average	%	16.079	7.16	-13.185

In the below tables, are values of APD90 (msec), dvdtmax (mV/msec) and APA (mV) from 4 animals (SP) for four different cycle lengths 200msec, 160msec, 120 msec and 80msec. As for the DP, while APD90 values show an overall increase across the 4 animals at different CL's, their corresponding APD (mV) and dvdtmax (mV/msec) show inconsistent results. This could be due to adaptation of the tissue to the cycle length.

Increase in APD90 durations ranged 5% to 25% for CL = 200 msec, 4% to 38% for CL = 160, average of 16% for CL =120 msec and about 26% for CL=80msec. This result shows that as the pacing rate increased, i.e. CL decreased, APD90 increased (green upward arrow) in presence of HG compared to Control.

Table 4: Average values of APD 90 durations (msec), APA (mV) and dvdtmax (mV/msec) taken from S2 beat paced with CL= 200msec from SP (n=4) showing average increase in all 4 animals for APD90 durations (msec), increase in 3 animals and decrease in 1 animal for APA (mV) and increase in 3 animals and decrease in 1 animal for dvdtmax (mV/msec).

Mouse #	Study	Durations	APA	dv/dt (max)
		APD90	(mV)	(mV/ms)
1	C	57.467	88.904	52.057
	HG	76.4	85.483	29.666
	%	24.78 ↑	-4	-75.47
3	C	45.533	81.638	44.66
	HG	56.633	96.259	56.728
	%	19.59 ↑	15.18 ↑	21.27 ↑
4	C	59.667	91.817	39.371
	HG	62.567	92.004	49.767
	%	4.63 ↑	0.2 ↑	20.88 ↑
5	C	66.9	65.897	21.27
	HG	84.433	94.936	45.133
	%	20.76 ↑	30.58 ↑	52.87 ↑
average	%	17.44	10.49	4.8875

Table 5: Average values of APD 90 durations (msec), APA (mV) and dvdtmax (mV/msec) taken from S2 beat paced with CL= 160msec from SP (n=4) showing average increase in all 4 animals for APD90 durations (msec), increase in 3 animals and decrease in 1 animal for APA (mV) and increase in 3 animals and decrease in 1 animal for dvdtmax (mV/msec).

Mouse #	Study	Durations	APA	dv/dt (max)
		APD90	(mV)	(mV/ms)
1	C	55.3	91.114	54.42
	HG	68.8	86.598	28.564
	%	19.622 ↑	-5.21	-90.57
3	C	45.233	82.854	44.727
	HG	57.367	97.379	56.249
	%	21.15 ↑	14.91 ↑	20.48 ↑
4	C	60.3	93.103	40.383
	HG	63.1	91.158	48.519
	%	4.437 ↑	-2.13 ↑	16.76 ↑
5	C	58.433	62.695	18.283
	HG	93.435	75.267	43.564
	%	37.46 ↑	16.7 ↑	58.03 ↑
average	%	20.66725	6.0675	1.175

Table 6: Average values of APD 90 durations (msec), APA (mV) and dvdtmax (mV/msec) taken from S2 beat paced with CL= 120 msec from SP (n=4) showing average increase in 3 animals and opposite effect in 1 animal for APD90 durations (msec), increase in 2 animals and decrease in 2 animals for APA (mV) and increase in 3 animals and decrease in 1 animal for dvdtmax (mV/msec).

Mouse #	Study	Durations APD90	APA (mV)	dv/dt (max) (mV/ms)
1	C	51.067	90.92	54.159
	HG	63.633	85.901	24.856
	%	19.74 ↑	-5.82	-117.8
3	C	45.3	83.603	44.509
	HG	55.867	96.997	55.31
	%	18.91 ↑	13.8 ↑	19.58 ↑
4	C	57.8	92.63	39.886
	HG	56.733	88.596	45.187
	%	-1.88	-4.55	11.73 ↑
5	C	52.167	59.624	15.224
	HG	70.833	93.667	43.406
	%	26.35 ↑	36.34 ↑	64.92 ↑
average	%	15.78	9.9425	-5.3925

Table 7: Average values of APD 90 durations (msec), APA (mV) and dvdtmax (mV/msec) taken from S2 beat paced with CL= 80 msec from SP (n=4) showing average increase in 3 animals and opposite effect in 1 animal for APD90 durations (msec), increase in 2 animals and decrease in 2 animals for APA (mV) and increase in 3 animals and decrease in 1 animal for dvdtmax (mV/msec).

Mouse #	Study	Durations	APA	dv/dt (max)
		APD90	(mV)	(mV/ms)
1	C	46.867	88.478	51.718
	HG	82.661	58.267	17.938
	%	43.3 ↑	-51.84	-188.3
3	C	44.067	83.565	43.206
	HG	53.1	97.252	53.42
	%	17.011 ↑	14.07 ↑	19.12 ↑
4	C	52.1	89.862	36.985
	HG	50.6	83.53	39.832
	%	-2.96	-7.5	7.14 ↑
5	C	47.233	55.042	11.541
	HG	86.912	58.467	33.071
	%	45.65 ↑	5.85 ↑	65.1 ↑
average	%	25.75025	-9.855	-24.235

In table 3 are the average APD's (msec) quantified from restitution beats across animals tested using both DP and SP. For Standard protocol (n=4) 3 animals showed an overall increase in APD ranging from 9% to 14% with an opposite effect seen in 1 animal (highlighted in red). Dynamic protocol (n=3) shows an average prolongation of 6% in duration in 2 of 3 animals with a decrease in other animals (highlighted in red).

Table 8: Average APD's (msec) quantified using CL-dependent changes between Control & HG. An overall increase in the APD was observed across the animals. Red highlighted boxes show the opposite effect of HG on APD. 'x' shows no data could be obtained in that trial for that animal.

Mouse #	Study	CL dependent changes (APD's in msec)	
		SP	DP
1	Control	47.78	51.832
	HG	55.17	50.73
	%	13.39	-2.17
3	Control	44.36	x
	HG	50.83	x
	%	12.73	
4	Control	47.06	63.1
	HG	44.32	63.89
	%	-6.18	1.23
5	Control	49.52	63.47
	HG	61.9	77.66
	%	20	18.27
average	%	9.985	5.776667

4.2 b Effect of HG on APD during Sinusoidal DI changes

To understand how the cell's action potential duration would vary when kept independent from Diastolic interval, the tissue was paced with explicit DI control pacing protocols. The center DI = 100msec was kept constant across the 4 pacing schemes. Two trials had a total duration of 240 beats with center DI=100 msec and varied sinusoidally ±20msec and ±30msec. The other two trials had a total duration of 220 beats and varied sinusoidally ±40 msec and ±50msec. Restitution was then quantified for each of these trials to see the effect of APD to its preceding DI and an example of the restitution curve obtained from Sin20-180 is plotted below (Figure 13). The restitution curves shown are APD values obtained for a single run (100 beats). Average duration in presence of HG was 20.76% more compared to Control.

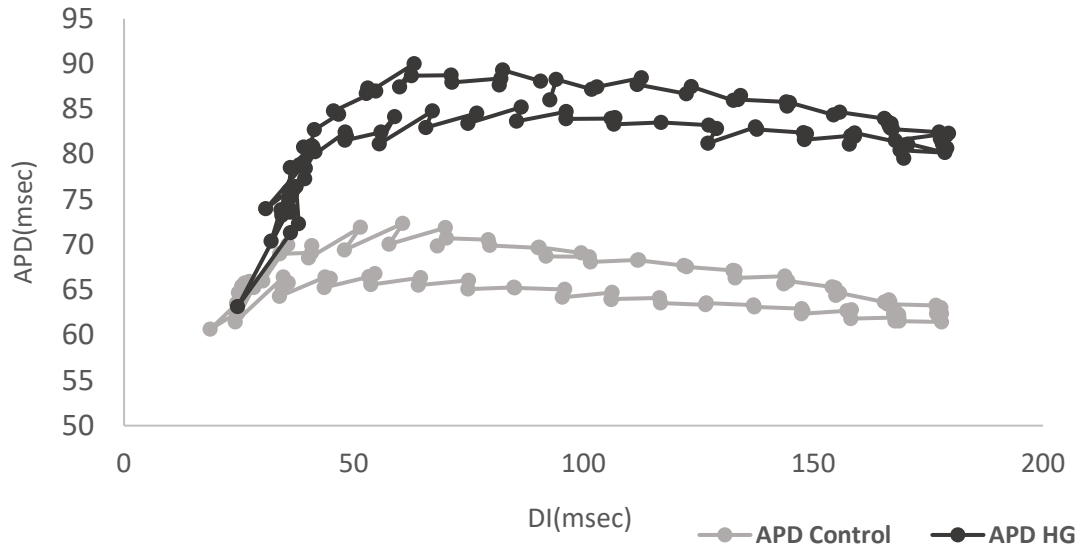


Figure 13 is an example of APD restitution curve obtained from Sin20-180 trial. The darker curve (HG) shows APD prolongation (mean APD= 81.88 msec) when compared to Control (Mean APD=64.88msec).

Average APD values were also quantified for all the trails across the 3 animals tested with Sinusoidal DI protocols. Table 4 shows APD in presence of HG compared to Control. The average APD prolongation ranged from 14% to 19% across the trials.

Table 9: Average APD (msec) values showing an increase in presence of HG compared to Control. The green upward arrow is the percentage increase in duration for each trial across the 3 animals.

Mouse #	**Study**	**Sinusoidal DI APD's**			
		Sin20-180	**Sin30-170**	**Sin40-160**	**Sin50-150**
1	**Control**	54.33	55.86	54.71	54.65
	HG	66.4	66.49	67.17	67.87
	%	18.17↑	15.99↑	18.55↑	19.48↑
4	**Control**	64.88	66.16	67.02	67.21
	HG	81.88	83.5	84.83	80.13
	%	20.76↑	20.76↑	20.99↑	16.12↑
5	**Control**	64.62	68.72	73.67	72.84
	HG	77.56	78.21	79.08	78.42
	%	16.68↑	12.13↑	6.84↑	7.11↑
average	**%**	18.54	16.29	15.46	14.23

4.2 c Effect of HG on APD70, APD 50 and APD 30 using CL- dependent changes

Early and late phase repolarizations were also computed as 30%, 50%, 70% and 90% recovery durations. Each of these repolarization levels have prominent ion channels regulating the duration and shape. When the tissue was subjected to acute HG buffer, with APD90 prolongation there was an overall increase in APD 70 supporting the

increase in late phase depolarization stage. However, early phase repolarization stage computed as APD 30 and APD50 showed inconsistent results.

The figures below are average APD's (msec) for all 4 repolarization stages. Every mouse was allotted a number which is used consistently throughout trials. Here, M1 stands for data from mouse 1; M4 for data from mouse 4 and M5 for data from mouse 5. Figure 14 are average changes in duration for dynamic restitution curves across the full run (410 beats) and Figure 15 are average changes in duration for standard restitution curves across the full run (502 beats).

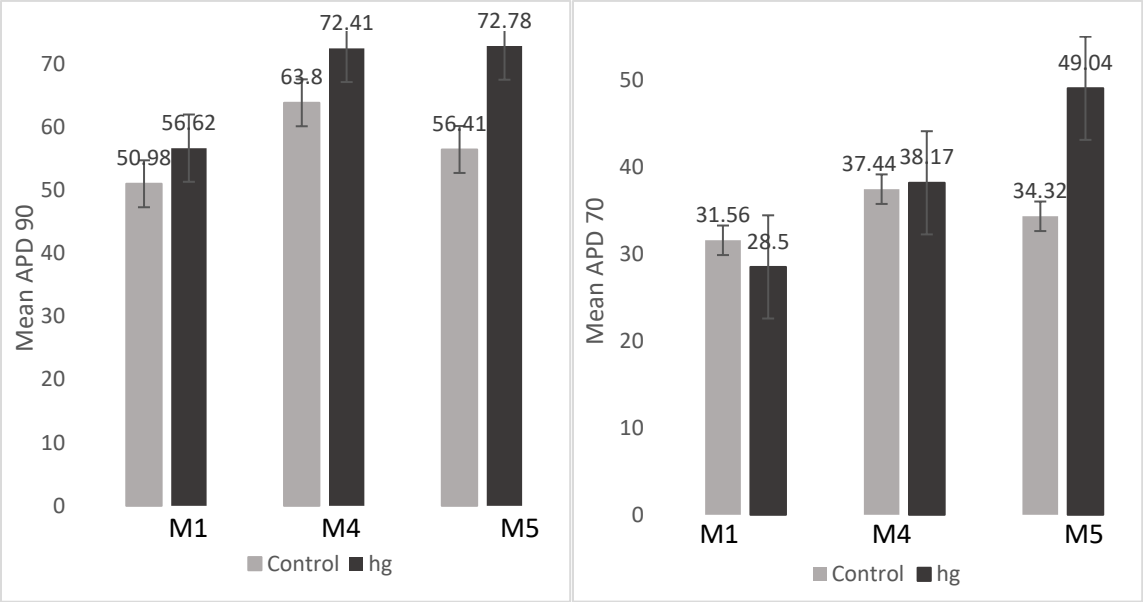


Figure 14.a: (Left-hand side) average APD 90 (msec) (n=3) showing an average increase in all 3 animals in presence of HG during DP. Figure 14.b: (Right-hand side) Average APD 70 (msec) (n=3) showing an average increase in 2 animals and a decrease in one animal in presence of HG during DP. M1, M4 and M5 are designated mice numbers allotted for every trial.

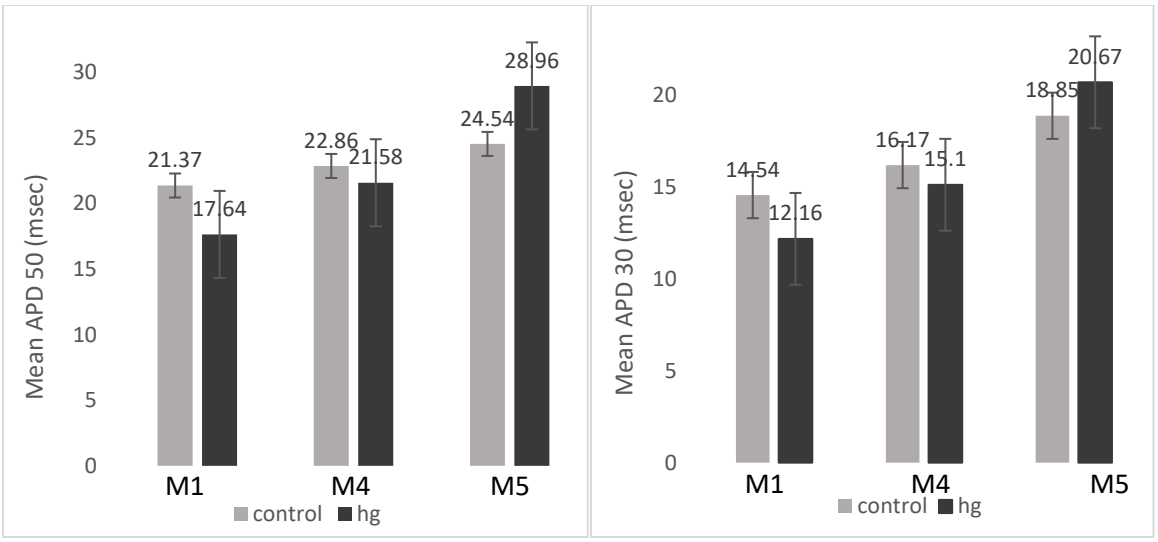


Figure 14.c: (Left-hand side) average APD 50 (msec) (n=3) showing an average decrease in 2 animals and increase in 1 animal in presence of HG during DP. Figure 14.d: (Right-hand side) average APD 30 (msec) (n=3) showing an average decrease in 2 animals and increase in 1 animal in presence of HG during DP.

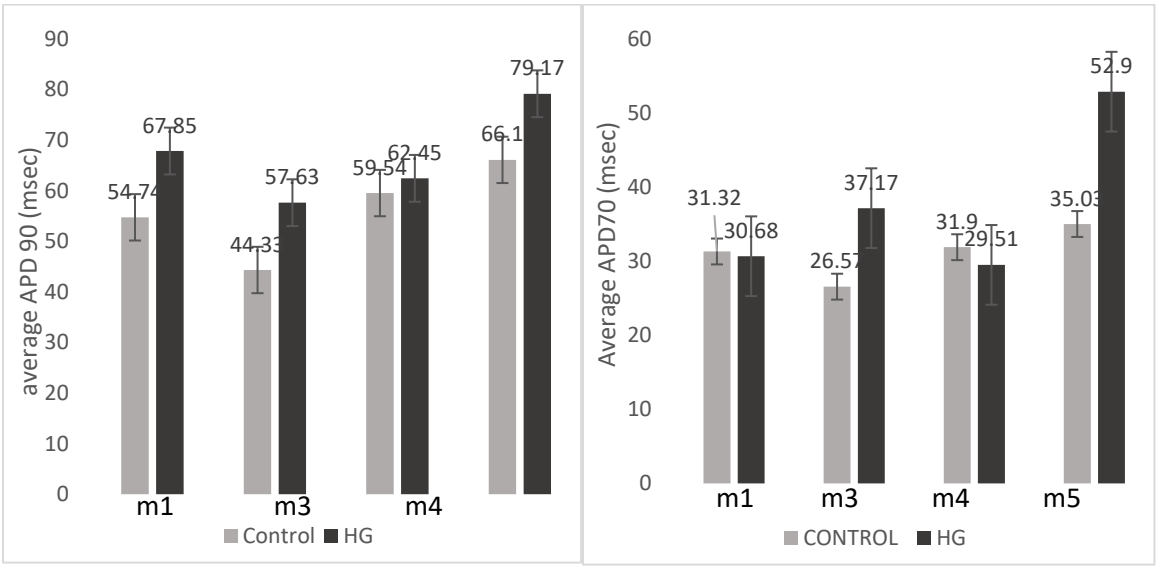


Figure 15.a: (Left-hand side) average APD 90 (msec) (n=4) showing an average increase in APD 90 in presence of HG during SP. Figure 15.b: (Right-hand side)

average APD 70 (msec) (n=4) showing an average increase in APD 70 in presence of HG in 2 animals and a decrease in the other two animals during SP.

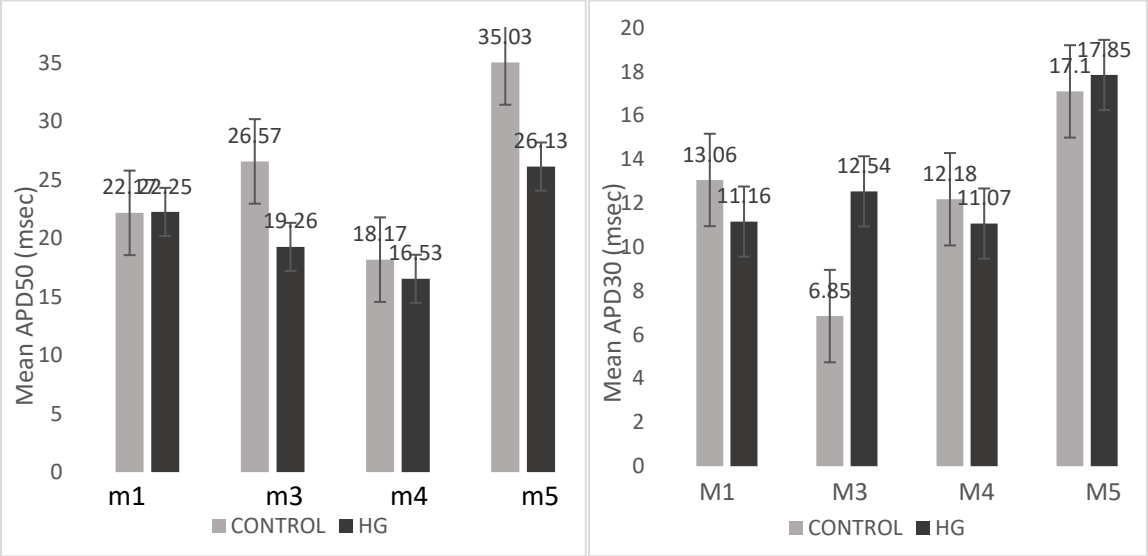


Figure 15.c: (Left-hand side) average APD 50 (msec) (n=4) showing an average decrease in APD 70 for 3 animals in presence of HG and an opposite effect in 1 animal during SP. Figure 15.d: (Right-hand side) average APD 30 (msec) (n=4) showing an average increase in 2 animals in presence of HG and a decrease in the other two animals during SP.

4.2 d Effect of HG on APD70, APD 50 and APD 30 using explicit diastolic interval (DI) control

The effects on APD at different levels of repolarization of HG are shown in table 5. While APD90 and APD70 showed prolongation, early phases of repolarization show opposite effects in the presence of HG.

The table 5 shows the effects of HG on APD70, APD50 and APD 30 when tissue was paced at different Sinusoidal pacing schemes; sin 20-180, sin 30-170, sin 40-160 and sin 50-150.

Table 10: Average durations at 70%, 50% and 30% repolarization (msec) of tissue in presence of HG. Positive differences in % show prolonging effects of HG on control and negative differences show opposite effects of HG on control.

mice #	Study	APD 70 (msec)				APD 50 (msec)				APD 30 (msec)			
		SIN 20-180	SIN 30-170	SIN 40 - 160	SIN 50-150	SIN 20-180	SIN 30-170	SIN 40 - 160	SIN 50-150	SIN 20-180	SIN 30-170	SIN 40 - 160	SIN 50-150
1	Control	31.26	31.86	31.48	31.32	20.26	20.56	20.3	20.06	13.17	13.34	12.05	15.62
	HG	32.58	31.7	31.45	31.45	19.02	18.32	18.16	18.16	12.2	11.74	11.59	11.59
	%	4.05	-0.5	-0.09	0.41	-6.51	-12.22	-11.78	-10.46	-7.95	-13.62	-3.96	-34.77
4	Control	34.99	36.5	34.73	34.72	19.75	20.13	20.16	19.96	12.97	13.59	13.59	13.41
	HG	37.65	37.34	35.72	37.1	19.54	19.45	19.68	19.49	13.4	13.4	13.58	13.14
	%	7.06	2.25	2.77	6.41	-1.07	-3.49	-2.43	-2.41	3.2	-1.41	-0.07	-2.05
5	Control	35.5	38.25	41.05	40.98	22.88	23.8	24.52	24.38	16.47	16.9	17.25	16.92
	HG	51.43	52.15	52.68	52.23	27.88	27.57	27.35	27.08	18.94	19.03	27.35	18.71
	%	30.97	26.65	22.07	21.53	17.93	13.67	10.34	9.97	13.04	11.19	36.92	9.56

Results in table 5 are consistent with the prolongation of APD 90 and show an average increase in APD70 for all the four pacing schemes. During the early phase of repolarization, which is APD 30 and APD50 durations, APD50 durations show an overall decrease in 2 out of 3 animals with an opposite effect in 1 animal (Mouse # 5; M5). This change in duration is observed across all the 4 pacing schemes for the animals. APD30 durations also show an inconsistent pattern across the animals. A decrease is observed in 2 out of 3 animals with an opposite effect in 1 animal (M5).

This animal (M5) showed the opposite effect and this was observed for several protocols run in this animal.

4.3 a. Effect of HG on action potential amplitude (APA)

Action potential amplitude (in mV) is the difference in TMP between that at the start of an AP to the peak (AP peak) of the AP.

During Sinusoidal DI control 2 of 3 animals showed a decrease in APA and one showed the opposite effect (M5). Of these two animals, 1 animal showed an average decrease of amplitude in presence of HG of 6%, the second animal showed an average decrease of amplitude in presence of HG of 11% while the third animal (M5) showed an average increase of amplitude in presence of HG of 32% (Figure 16).

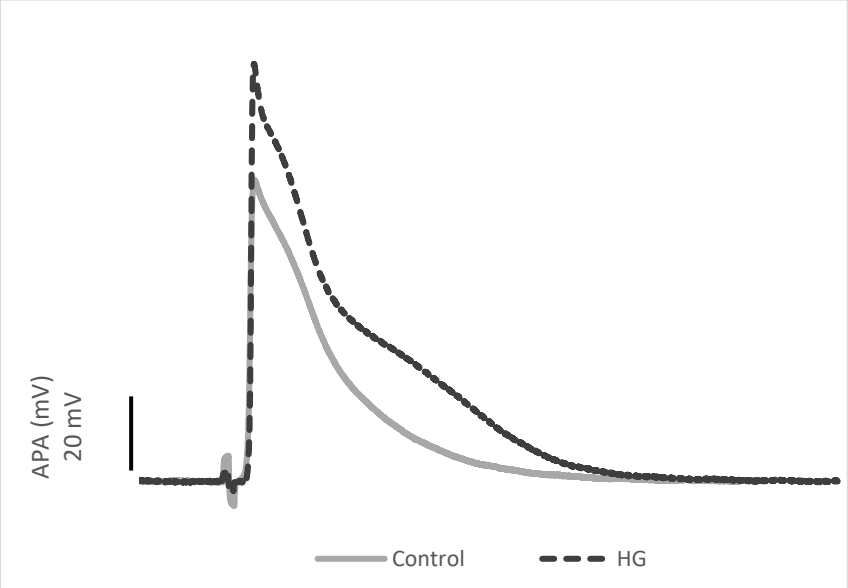


Figure 16: Example of an AP with increase in APA (32%) in presence of HG compared to control.

During CL-dependent changes (SP and DP) there was an overall decrease in APA in 2 of 4 animals (M1 and M4) and an increase in the other two (M3 and M5).

4.3 b. Effect of HG on maximum rate of depolarization (dvdtmax)

In presence of HG the upstroke velocity varied between animals. Like the APA variations in Sinusoidal DI protocols, dvdtmax (n=3) across the pacing schemes decreased in 2 of 3 animals and an opposite effect was seen in 1 animal (M5).

During DP, 2 of 3 animals showed an increase in dvdtmax in presence of HG comparing to control (M4 and M5) and one animal showed a decrease (M1). During SP, 3 of 4 animals showed an increase in dvdtmax (M3, M4 and M5) and one animal (M1) showed a decrease. Figure 17 shows the average dvdtmax (mV/msec) to increase in 3 of 4 animals during SP in presence of HG (mean APA= 45.28 mV) compared to Control (mean APA= 41.71 mV).

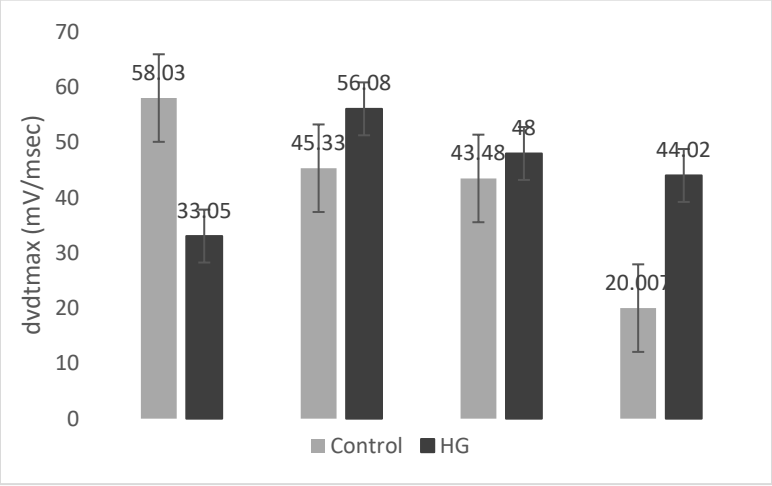


Figure 17: Average dvdtmax (mV/msec) in presence of HG (mean APA= 45.28 mV) compared to control (mean APA= 41.71 mV) for 4 animals showing increase of dvdtmax in 3 animals and opposite effect in 1 animal.

4.4 Effect of HG on alternans

Alternans are beat to beat changes in electrophysiological features. In this study, alternans was computed as beat to beat changes in APD at different levels of repolarization (90%, 70%, 50% and 30%), in APA and in dvdtmax.

4.4 a. *Effect of HG on alternans when tissue is paced with CL- dependent changes*

Data from 1 animal (M5) showed incidences of in-phase alternans, i.e. within a long-short APD sequence the long APDs were accompanied by larger dvdtmax and shorter APDs were accompanied by smaller dvdtmax. Using the Dynamic restitution protocol and during HG in-phase alternans was seen in one animal (M5) except that, in the same animal (M5), there were 5 beats of out of phase alternans between dvdtmax and APD, i.e. the long APD of long-short was accompanied by smaller dvdtmax and vice versa. After this run of 5 beats, activation was blocked (i.e. the stimulus failed to produce an AP). Figure 18 shows the out of phase alternans from animal M5.

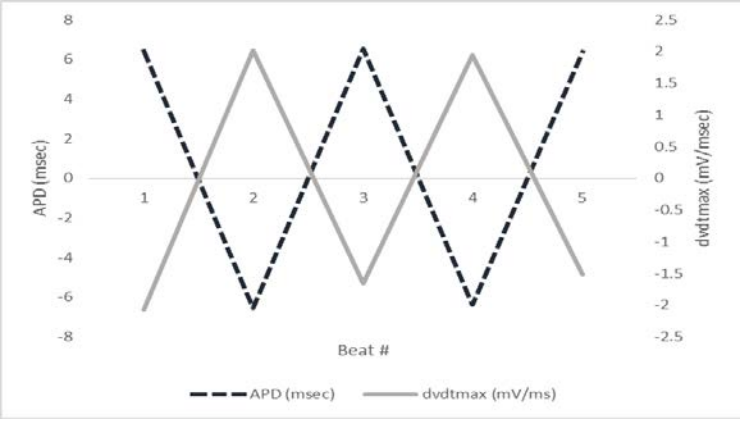


Figure 18: An instance of out-of- phase behavior between APD and dvdtmax in presence of HG for 5 beats. Shown on the axes are differences in APD in msec (left) and in dvdtmax (right).

Apart from these changes, HG had effects on alternans by inducing the first occurrence of alternans at a much slower activation rate (longer CL) than Control. The instances when alternans threshold, i.e. CL when alternans is first seen, changed well few and therefore a comparison in terms of effects on alternans threshold cannot be made.

Alternans threshold is the longest (slowest) pacing rate at which the first APD beat to beat fluctuations are observed. Alternans threshold was investigated during both DP and SP to see if HG produced first alternans at a longer cycle length, but the results showed no effect of HG on alternans threshold.

4.4 b. Effect of HG on alternans when tissue is paced with Sinusoidal DI changes

Tissue paced at different DI protocols showed almost no effect of HG on alternans. Most of the recorded datasets had no alternans either during Control or HG. Few trials showed, however, that when Control produced few beats of APD90 alternans, addition of HG made the tissue more stable by not inducing alternans that is, alternans was seen during control but not after addition of HG.

4.4 c. Effect of HG on alternans for different levels of repolarization stages (70%, 50% and 30%)

For 3 animals tested for APD70 alternans only one animal (M5) showed APD 70 alternans when tissue was paced with Sin DI 20-180, Sin DI 30-170 and Sin DI 40-160. However, there were instances during trials where addition of HG induced alternans. For example, during Sin 20-180 and Sin 30-170, Control buffer produced no alternans but, addition of HG induced 2% - 3% of alternans for that tissue. During

Sin 40-160 when animal M5 showed instances of alternans in presence of Control in 1.8% of the beats, addition of HG to the tissue further induced 2.2% of alternating beats. Animal M5 only showed that HG influenced alternans at 70% repolarizing stage.

There was no evidence of APD 50 and APD 30 alternans throughout the data for tissue paced with Sinusoidal DI protocol. Despite these results, the differences are small enough to conclude that HG had no effect on alternans. Moreover, HG showed no effect on alternans at the early phases of repolarization.

4.5 Effect of HG on constant DI

Constant DI studies were performed to see the effect of HG on alternans. From the data collected (n=3) there were few instances that showed addition of HG induced alternans of APD, APA and dvdtmax. In animal M1 when control produced no APD alternans, addition of HG caused 14% of alternating beats under constant DI=20msec. In animal M4, HG induced 15% to 22% of alternating APD beats. But these account for few instances and do not imply that HG by itself has any prominent effect on alternans. Overall, even though the instances at which HG induced alternans were very few its effect on alternans was not profound.

Summary from Part I

From the above results, addition of HG prolonged the late phase repolarization durations by increasing APD and APD70. APA in presence of HG decreased compared to Control. Effect of HG on upstroke velocity was not clear due to mixed results. Moreover, effect of HG on alternans was not conclusive. When addition of HG induced

almost no alternans in 2 out of 3 animals, in one animal decreased the number of alternating beats making the tissue more stable.

Part 2

4.5a Effect of Isoproterenol (ISO) on Control using Sin DI pacing schemes (n=3)

Effects of Isoproterenol were studied to understand electrophysiological changes to the tissue. Included in table 6 are action potential durations (APD90) between Control and ISO when tissue was paced with Sinusoidal DI protocols (n=3). All animals showed a decrease in APD90 (msec) in presence of ISO with an average decrease of 17.59% across the 4 Sinusoidal DI pacing schemes.

Table 11: Average APD (msec) showing a decrease in presence of ISO ranging from 10% to 26%. The red downward arrow are the percentage decreases in durations for each trial across the 3 animals except for Sin 20-180 values for Mouse #2 where, readings for control was not collected as the run was not complete.

Mouse #	Study	Sinusoidal DI APD's			
		Sin20-180	Sin30-170	Sin40-160	Sin50-150
2	Control		79.44	81.52	83.25
	ISO	58.98	57.52	56.61	53.26
	%		38.1 ↓	44 ↓	56.3 ↓
4	Control	64.95	66.17	67.02	67.21
	ISO	55.86	58.9	58.77	60.35
	%	16.27 ↓	12.34 ↓	14.03 ↓	11.36 ↓
5	Control	64.58	68.71	73.67	72.83
	ISO	61.61	64.23	63.74	65.66
	%	4.82 ↓	6.97 ↓	15.57 ↓	10.91 ↓
average	%	10.545	19.13	24.53	26.19

4.5b Effect of Isoproterenol (ISO) using CL-dependent pacing schemes (n=4)

Similar decrease in APD durations in presence of ISO were observed in CL-dependent pacing protocols (SP and DP). The table below (table 7) shows the average durations (S1-S2 and S1-S1) for control and ISO. For SP(n=4), except for one animal that showed an opposite effect (increase in APD) the remaining 3 animals shows an APD decrease ranging from 21% to 50%. Dynamic protocol (n=4) studies gave an average decrease of 17.3% in APD90 durations (msec) across the animals in presence of ISO compared to Control.

Table 12: Average APD's (msec) quantified using CL-dependent changes between Control & ISO. Red downward arrows showed the decreased effect of ISO on APD. 'x' shows no data was obtained in that trial for that animal.

Mouse #	Study	Sinusoidal DI	
		SP	DP
O	Control	no data	69.38
	ISO	no data	65.353
	%		6.16 ↓
2	Control	66.967	74.915
	ISO	45.235	50.146
	%	48.04 ↓	49.39 ↓
3	Control	44.367	no data
	ISO	52.606	no data
	%	15.66 ↑	
4	Control	47.068	63.101
	ISO	38.957	55.626
	%	20.82 ↓	13.43 ↓
5	Control	61.901	63.474
	ISO	41.417	63.277
	%	49.45 ↓	0.311 ↓
average	%	33.49	17.32

An example of the restitution curves obtained from DP (S1-S1 beats) is shown below (figure 19). The average decrease of APD in presence of ISO was 13.43%.

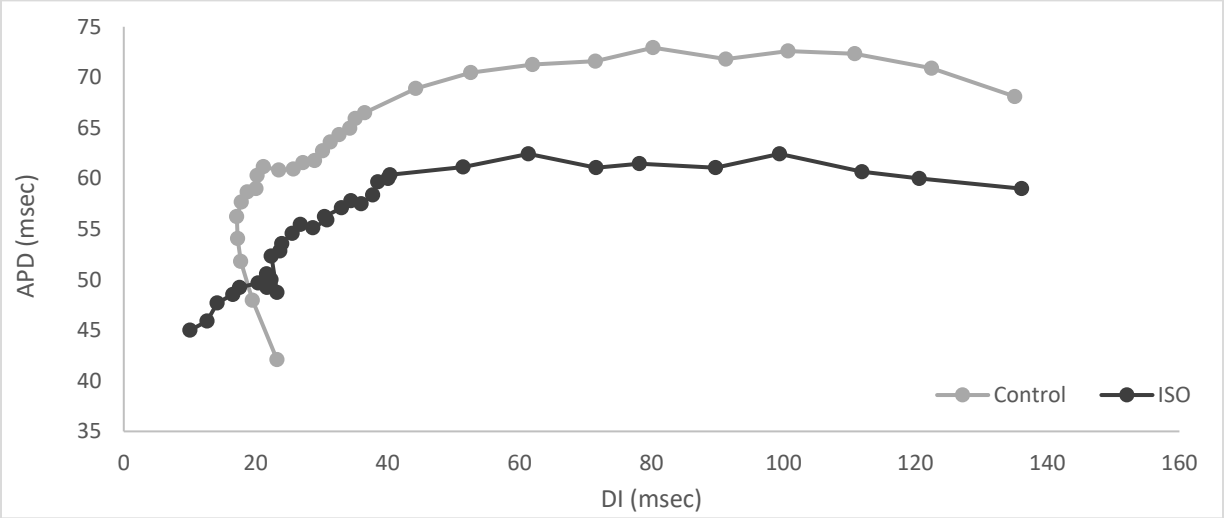


Figure 19: Dynamic restitution curve (S1- S1) showing APD decrease in presence of ISO (dark) compared to Control (light). The average decrease of APD in presence of ISO was 13.43%.

From the above results, it is seen that the effect of ISO was to decreases the action potential duration in majority of the trials.

4.5 c Effect of Isoproterenol on action potential amplitude (APA)

Addition of Isoproterenol increased APA enhancing its effects on inward sodium current (Ina+) by increasing the influx of sodium ions increasing the amplitude. APA increase was observed in 2 out of 3 animals. Figure 20 shows an example of average APA (mV) quantified from animals paced with Sin DI 30-170 (n=3). Similar increase in APA (mV) was observed in 2 animals (M2 & M5) during the Sin DI protocols.

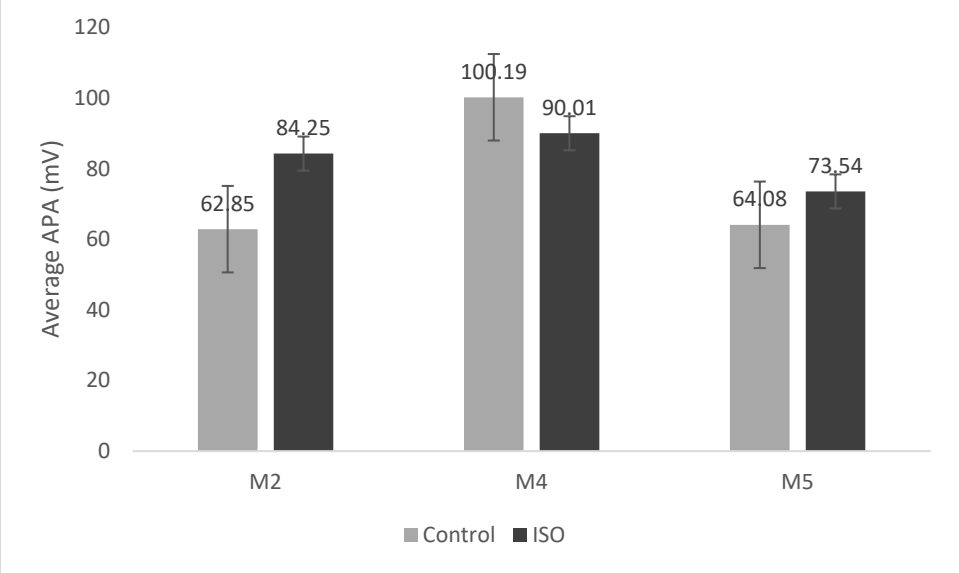


Figure 20: Average APA (mV) values quantified from 3 animals paced with center DI= 100msec with a range of ± 70 msec showing an overall increase of APA in presence of ISO in 2 out of 3 animals and an opposite effect in 1 animal.

4.5 d Effect of Isoproterenol on dvdtmax (mV/msec) (n=3)

From the DI protocols, addition of ISO (n=3) to the buffer increased the upstroke velocity in 2 of 3 animals (M2 & M4). The last animal (M5) showed an increase in dvdtmax (mV/msec) when tissue was paced using Sin 40-160 and Sin 50-150 but not for all trials. The overall increase of dvdtmax in presence of ISO ranged 11% to 73% across the animals paced for different protocols.

Summary from Part II

Addition of ISO to the buffer decreased the APD when tissue was paced with both CL and DI protocols. Also, with ISO an overall increase in both APA and dvdtmax was observed when compared to control.

Part 3

4.6 Effect of HG on Isoproterenol (ISO)

The β-adrenergic agonist, Isoproterenol (ISO), was added to the buffer to mimic adrenergic activity.

4.6 a. AP characteristics between HG & ISO

Figure 21 shows APD prolongation in presence of HG compared to ISO. The TMP's were recorded at CL=180 msec from animal M5 with an average increase of 8% in presence of HG (dark dotted line) compared to ISO (light solid line).

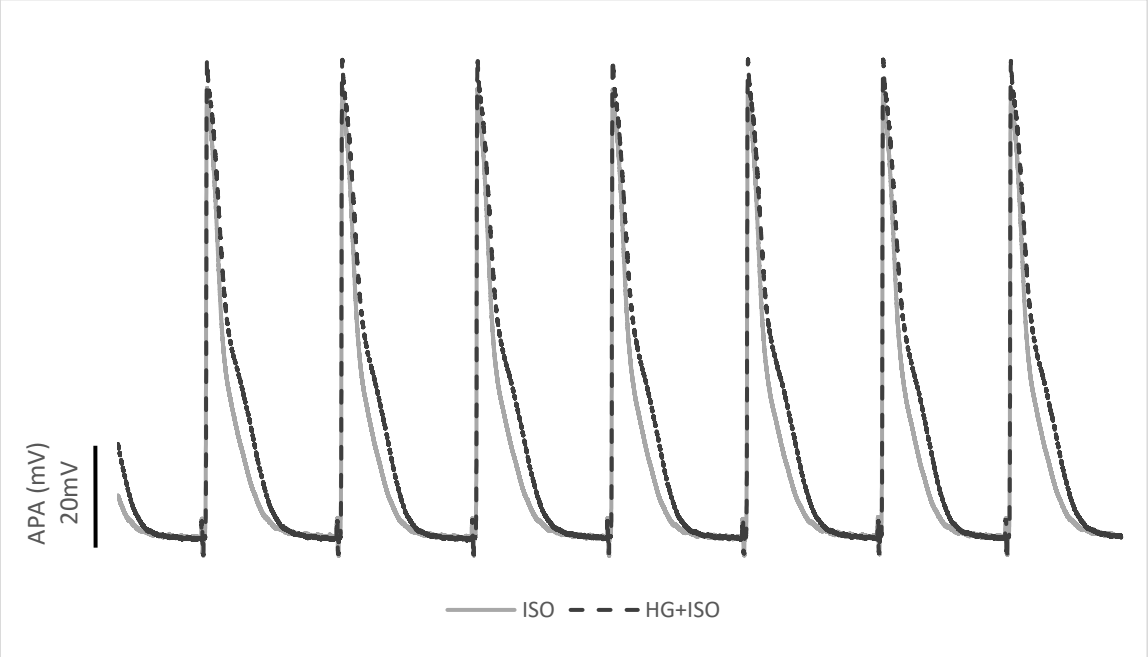


Figure 21: Transmembrane potentials showing APD prolongation along with an increase in APA in presence of HG compared to ISO at CL= 180ms. APD prolongation was at an average of 8% in presence of HG.

4.7 Effect of HG on APD when added to ISO

Animals (n=5) were paced with both CL-dependent changes (SP & DP) and sinusoidal DI protocols when the tissue was superfused first with ISO and then HG was added. An overall increase in APD (msec) was observed in presence of HG for 4 animals, an opposite effect was observed in one animal.

4.7 a Effect of HG on APD when added into ISO during CL-dependent changes

Tissue paced at different activation rates as explained using dynamic (S1-S1) and Standard protocol (S1-S2) showed an APD prolongation in presence of HG. This increase ranged 3% to 14% using DP (n=5) and 4% to 11 % using SP (n=4). Given in table 8 are the average durations from the restitution beats (DP & SP) between ISO and HG.

Table 13: Average APD's (msec) quantified using CL-dependent changes between ISO & HG. An overall increase in the APD was observed across the animals. The average increase in APD in presence of HG and ISO (HG+ISO) was 5% for DP and 2.3% for SP. Red highlighted boxes are the opposite effect of HG on APD. 'x' shows no data was obtained in that trial for the animal.

Mouse #	Study	Sinusoidal DI	
		SP	DP
O	ISO	x	65.353
	HG+ISO	x	67.363
	%		2.98
2	ISO	45.24	50.146
	HG+ISO	47.24	58.37
	%	4.23	14.0894295
3	ISO	52.6	58.52
	HG+ISO	49.44	59.23
	%	-6.39	1.2
4	ISO	38.96	54.96
	HG+ISO	43.91	56.97
	%	11.27	3.53
5	ISO	41.41	63.277
	HG+ISO	41.43	65.45
	%	0.05	3.32
average	%	2.29	5.0238859

For SP (n=4) while 3 animals showed APD prolongation in presence of HG when ISO was already there, one animal (M3) showed a further decrease in APD in presence of

HG (opposite effect). This is the same animal that showed an increase in APD in presence of ISO (see table 7 M3; SP data).

Figure 22.1 shows an example of a standard restitution curve from an animal whose duration increased in presence of HG (dark) when compared to ISO (light). The average increase of APD is 4.23% (M2).

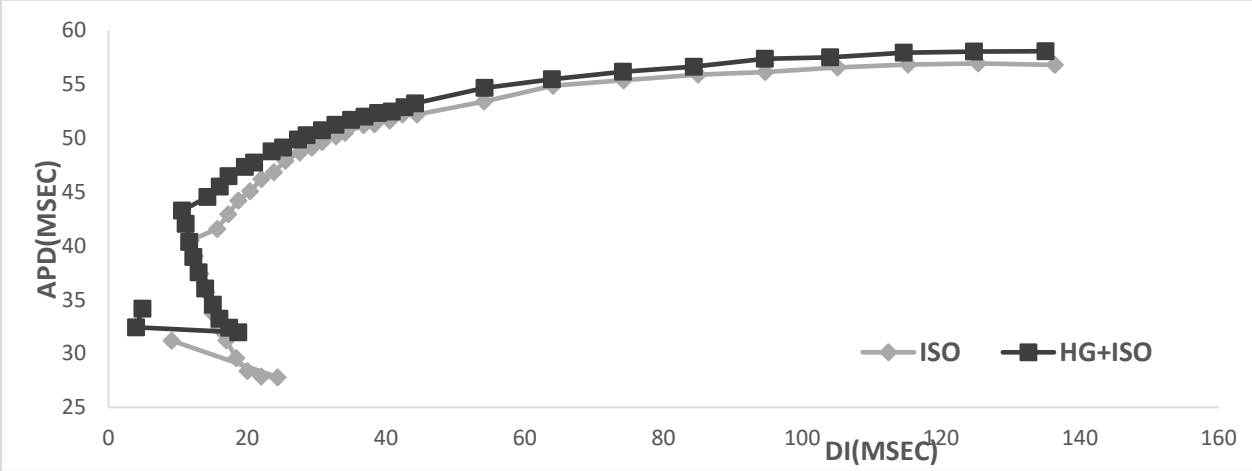


Figure 22.a: Standard restitution curve (S1-S1) showing APD prolongation in presence of HG compared to ISO. The average increase of APD was 4.23% (M2).

The above figure also shows an example of adaptation. As the CL (activation rate) decreased there was a decrease in APD and DI. Towards the end of the trial as the CL decreased there was a decrease in APD but with an increase in DI. This example showed that the tissue begins to adapt to the fast pacing rates by increasing the duration of DI.

An example of the dynamic restitution curve (n=5) depicting APD prolongation in presence of HG with an average increase of 14% is shown below (M2). The figure shows that APD in presence of HG had increased in while ISO was also present.

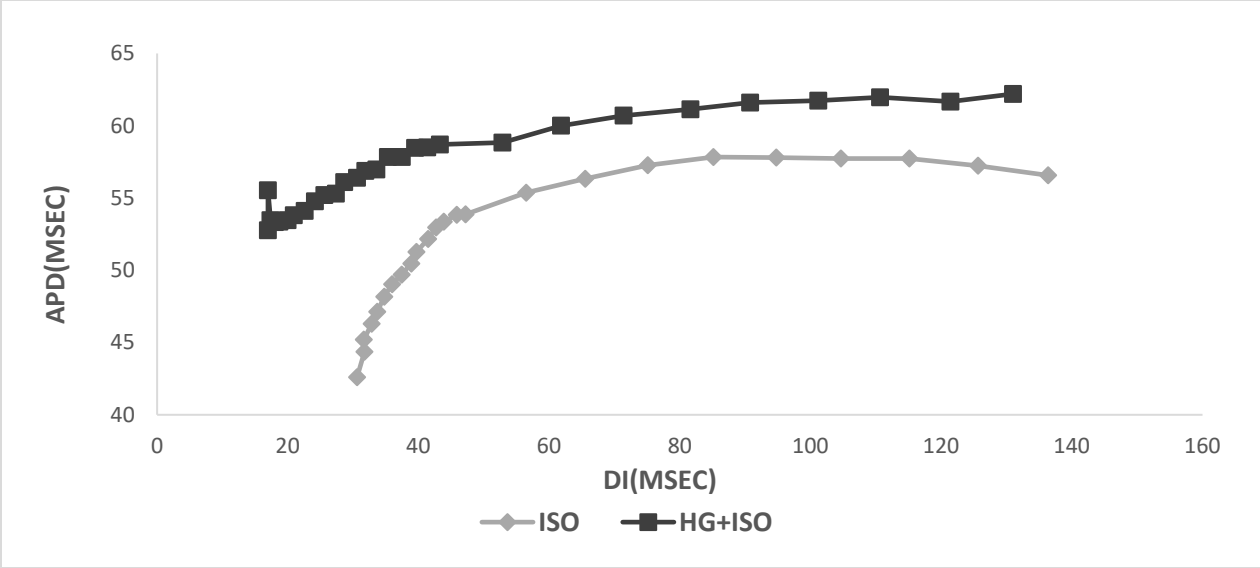


Figure 22.b: Dynamic restitution curve (S1-S1) from M2 showing APD prolongation in presence of HG compared to ISO with an average increase of 14%

Given in table 9 are values of APD90, dvdtmax (mV/msec) and APA (mV) from 5 animals (DP) for three different cycle lengths 200msec, 160msec and 120 msec. When APD90 values increased for three different CL's in 4 of 5 animals with an opposite effect (decrease in APD90) in 1 animal, their corresponding APA (mV) and dvdtmax (mV/msec) also increased in these 4 animals in the presence of HG compared to results during ISO and opposite effect in 1 animal.

Increase in APD90 durations averaged 4.4% for CL = 200msec, 9% for CL = 160 msec and 7.4% for CL =120 msec.

Table 14: Average values of APD 90 durations (msec), APA (mV) and dvdtmax (mV/msec) taken from 10 beats (S1) paced with CL= 200msec from DP (n=5) showed an average increase in all 5 animals for APD90 durations (msec), increase in 4 animals and decrease in 1 animal for APA (mV) and increase in 4 animals and decrease in 1 animal for dvdtmax (mV/msec).

Mouse #	Study	Durations	APA	dv/dt (max)
		APD90	(mV)	
0	ISO	75.64	92.23	38.986
	HG+ISO	78.912	96.69	42.125
	%	4.15 ↑	4.61 ↑	7.45 ↑
2	ISO	55.727	91.928	52.77
	HG+ISO	59.08	102.34	80.247
	%	5.67 ↑	10.17 ↑	34.24 ↑
3	ISO	61.853	88.262	48.295
	HG+ISO	61.99	96.384	56.567
	%	0.221 ↑	8.42 ↑	14.62 ↑
4	ISO	62.127	97.142	66.641
	HG+ISO	63.07	81.095	41.498
	%	1.49 ↑	19.8 ↓	60.5 ↓
5	ISO	72.489	89.444	31.785
	HG+ISO	80.811	93.97	35.315
	%	10.29 ↑	4.81 ↑	9.99 ↑
average	%	4.3642	7.0025	16.575

Table 15: Average values of APD 90 durations (msec), APA (mV) and dvdtmax (mV/msec) taken from 10 beats (S1) paced with CL= 160msec from Dynamic protocol (n=5) showed an average increase in all 3 animals for APD90 durations (msec), increase in 3 animals and decrease in 1 animal for APA (mV) and increase in 4 animals and decrease in 1 animal for dvdtmax (mV/msec).

Mouse #	Study	Durations APD90	APA (mV)	dv/dt (max)
0	ISO	73.227	90.14	39.284
	HG+ISO	78.013	93.84	40.466
	%	6.14 ↑	3.94 ↑	2.92 ↑
2	ISO	58.023	91.849	50.984
	HG+ISO	62.483	101.89	76.887
	%	7.137 ↑	9.85 ↑	33.68 ↑
3	ISO	64.983	88.077	45.907
	HG+ISO	63.977	96.051	54.938
	%	-1.57	8.3 ↑	16.45 ↑
4	ISO	67.25	97.39	66.097
	HG+ISO	64.861	82.054	42.229
	%	-238.9	-1533.6	-2386.8
5	ISO	70.364	89.397	28.972
	HG+ISO	81.609	90.532	31.547
	%	13.77 ↑	1.25 ↑	8.16 ↑
average	%	9.015666667	5.835	15.3025

Table 16: Average values of APD 90 durations (msec), APA (mV) and dvdtmax (mV/msec) taken from 10 beats (S1) paced with CL= 120msec from DP (n=5) showed an average increase in 4 animals for APD90 durations (msec), increase in 4 animals and decrease in 1 animal for APA (mV) and increase in 4 animals and decrease in 1 animal for dvdtmax (mV/msec).

Mouse #	Study	Durations APD90	APA (mV)	dv/dt (max)
0	ISO	69.103	87.75	38.72
	HG+ISO	74.87	90.46	39.211
	%	7.7 ↑	2.99 ↑	1.25 ↑
2	ISO	56.87	91.554	47.66
	HG+ISO	60.187	101.12	72.157
	%	5.51 ↑	9.46 ↑	33.94 ↑
3	ISO	65.053	90.565	41.827
	HG+ISO	63.223	95.871	52.16
	%	2.89 ↓	5.53 ↑	19.81 ↑
4	ISO	65.797	97.368	64.875
	HG+ISO	66.458	82.652	42.13
	%	0.99 ↑	17.80 ↓	53.98 ↓
5	ISO	65.5	81.247	24.691
	HG+ISO	77.352	84.925	25.198
	%	15.33 ↑	4.33 ↑	2.01 ↑
average	%	7.3825	5.593333	12.4

Table 10, showed APD90 (msec), dvdtmax (mV/msec) and APA (mV) from 4 animals (SP) for four different cycle lengths 200msec, 160msec, 120 msec and 80msec. When APD90 (msec) values showed an overall increase across the 4 animals at different CL's, their corresponding APD (mV) and dvdtmax (mV/msec) showed inconsistent results.

Increase in APD90 durations ranged 5% to 25% for CL = 200msec, 4% to 38% for CL = 160, average of 16% for CL =120 msec and about 26% for CL=80msec.

Table 17: Average APD 90 durations (msec), APA (mV) and dvdtmax (mV/msec) taken from beat paced at CL= 200msec from SP (n=4) showed an average increase in 3 animals for APD90 durations (msec), increase in all animals for APA (mV) and increase in 3 animals and decrease in 1 animal for dvdtmax (mV/msec).

Mouse #	Study	Durations	APA	dv/dt (max)
		APD90	(mV)	
2	ISO	56.033	97.935	66.609
	HG+ISO	56.667	103.87	83.105
	%	1.1 ↑	5.71 ↑	19.84 ↑
3	ISO	55.4	89.382	52.384
	HG+ISO	54.033	99.422	60.126
	%	-2.52	10.09 ↑	12.87 ↑
4	ISO	45.1	83.764	54.783
	HG+ISO	52.7	85.258	46.102
	%	14.42 ↑	1.75 ↑	-18.83
5	ISO	60.2	68.9	14.085
	HG+ISO	79.6	87.854	31.363
	%	24.37 ↑	21.57 ↑	55.09 ↑
average	%	13.2966667	9.78	17.2425

Table 18: Average values of APD 90 durations (msec), APA (mV) and dvdtmax (mV/msec) taken from beat paced at CL= 160msec from SP (n=4) showed an average APD90 increase in 3 animals and decrease in 1 animal; increase in 3 animals and decrease in 1 animal for APA (mV) and increase in 3 animals and decrease in 1 animal for dvdtmax (mV/msec).

Mouse #	Study	Durations	APA	dv/dt (max)
		APD90	(mV)	
2	ISO	56.567	97.637	64.882
	HG+ISO	57.5	103.56	81.748
	%	1.62 ↑	5.71 ↑	20.63 ↑
3	ISO	58.567	89.981	49.331
	HG+ISO	55.3	99.774	59.92
	%	-5.9	9.81 ↑	17.67 ↑
4	ISO	48.6	85.427	57.237
	HG+ISO	55.233	85.36	47.271
	%	12 ↑	-0.07	-21.08
5	ISO	55.233	68.29	13.655
	HG+ISO	75.367	86.301	29.382
	%	26.71 ↑	20.87 ↑	53.52 ↑
average	%	13.44333333	12.13	30.60667

Table 19: Average values of APD 90 durations (msec), APA (mV) and dvdtmax (mV/msec) taken from beat paced at CL= 120msec from SP (n=4) showed an average APD90 increase in 3 animals and decrease in 1 animal; increase in 3 animals and decrease in 1 animal for APA (mV) and increase in 3 animals and decrease in 1 animal for dvdtmax (mV/msec).

Mouse #	Study	Durations APD90	APA (mV)	dv/dt (max)
2	ISO	55.367	97.517	63.065
	HG+ISO	56.167	103.39	80.409
	%	1.42 ↑	5.68 ↑	21.56 ↑
3	ISO	59.233	92.988	48.131
	HG+ISO	54.3	99.974	59.333
	%	-9.08	6.98 ↑	18.87 ↑
4	ISO	44.267	86.079	56.025
	HG+ISO	51.633	81.635	45.133
	%	14.26 ↑	-5.44	-24.13
5	ISO	51.167	68.763	12.97
	HG+ISO	68.267	84.478	27.376
	%	25.04 ↑	18.6 ↑	52.62 ↑
average	%	13.57	10.42	31.01667

Table 20: Average values of APD 90 durations (msec), APA (mV) and dvdtmax (mV/msec) taken from 10 beats paced with CL= 80msec from SP (n=4) showed an average APD90 increase in 3 animals and decrease in 1 animal; increase in 3 animals and decrease in 1 animal for APA (mV) and increase in 3 animals and decrease in 1 animal for dvdtmax (mV/msec).

Mouse #	Study	Durations	APA	dv/dt (max)
		APD90	(mV)	
2	ISO	52.2	95.676	55.225
	HG+ISO	52.867	102.3	75.175
	%	1.26 ↑	6.47 ↑	26.53 ↑
3	ISO	56.7	92.189	43.2
	HG+ISO	52.533	100.37	60.108
	%	-7.93	8.15 ↑	28.12 ↑
4	ISO	45.667	85.087	52.942
	HG+ISO	47.333	78.01	40.31
	%	3.52 ↑	-9.07	-31.33
5	ISO	42.133	61.6	8.4813
	HG+ISO	60.167	77.316	19.543
	%	29.97 ↑	20.32 ↑	56.601 ↑
average	%	11.583	11.64	37.08

4.7 b Effect of HG on APD when added into ISO during Sinusoidal DI changes (n=4)

With center DI = 100 msec, the tissue was paced at different Sin DI to see the effect of HG on ISO. From 4 animals of the group, 3 showed APD prolongation with HG with an average of 3% to 7% across pacing schemes. One animal showed an opposite effect where APD decrease in presence of HG and ISO (HG+ISO). Table 11 shows the average APD increase / decrease for n=4 across Sin DI protocols.

Table 21: Average APD (msec) showed an increase in presence of HG when added into ISO. The green upward arrow is the percentage increase in APD for across the 3 animals with an average increase of 3% to 7%.

Mouse #	Study	Sinusoidal DI APD's			
		Sin20-180	Sin30-170	Sin40-160	Sin50-150
2	ISO	58.97	57.53	56.6	58.63
	HG+ISO	61.5	61.6	61.32	61.29
	%	4.11↑	6.6↑	7.69↑	4.34↑
3	ISO	61.09	68.63	61.36	61.02
	HG+ISO	58.64	60.89	59.42	59.7
	%	4.17↓	12.71↓	3.23↓	2.21↓
4	ISO	55.86	58.9	58.77	60.35
	HG+ISO	60.29	59.92	59.94	60.71
	%	7.34↑	1.7↑	1.95↑	0.59↑
5	ISO	61.61	64.23	63.74	65.66
	HG+ISO	77.29	76.46	78.26	78.92
	%	20.28↑	15.99↑	18.55↑	16.8↑
average	%	6.89	2.89	6.23	4.88

An example of APD restitution curve from an animal paced using SIN DI 20-180 protocol is shown below (Figure 23). The average increase in APD90 in presence of

HG was 4.11% (M2). This figure represents APD restitution curve from 1 period (100 beats), 1st of the two cycles. APD prolongation was seen in 3 out of 4 animals that showed a restitution curve as shown below.

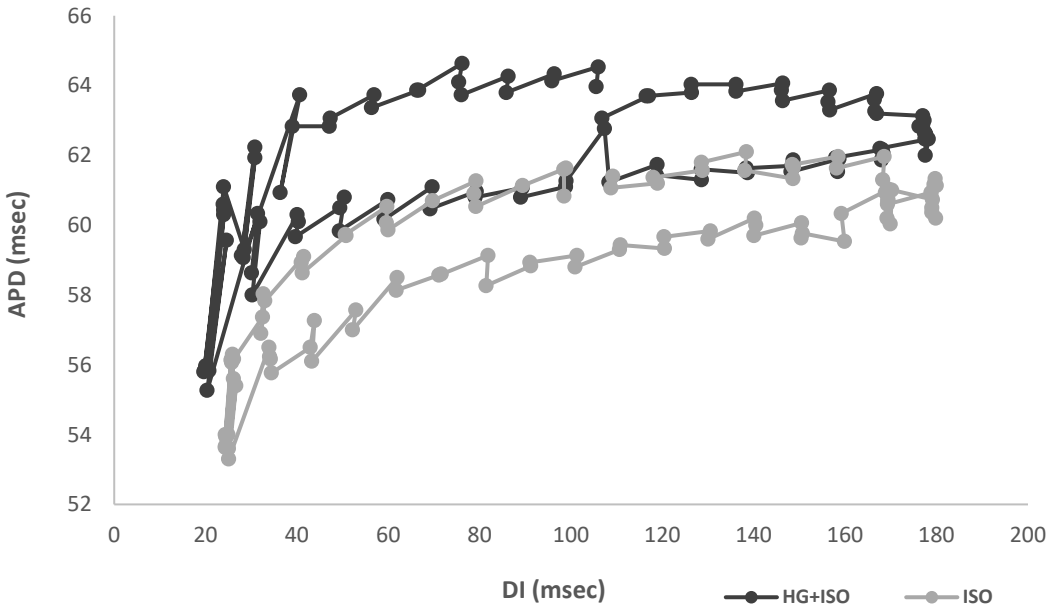


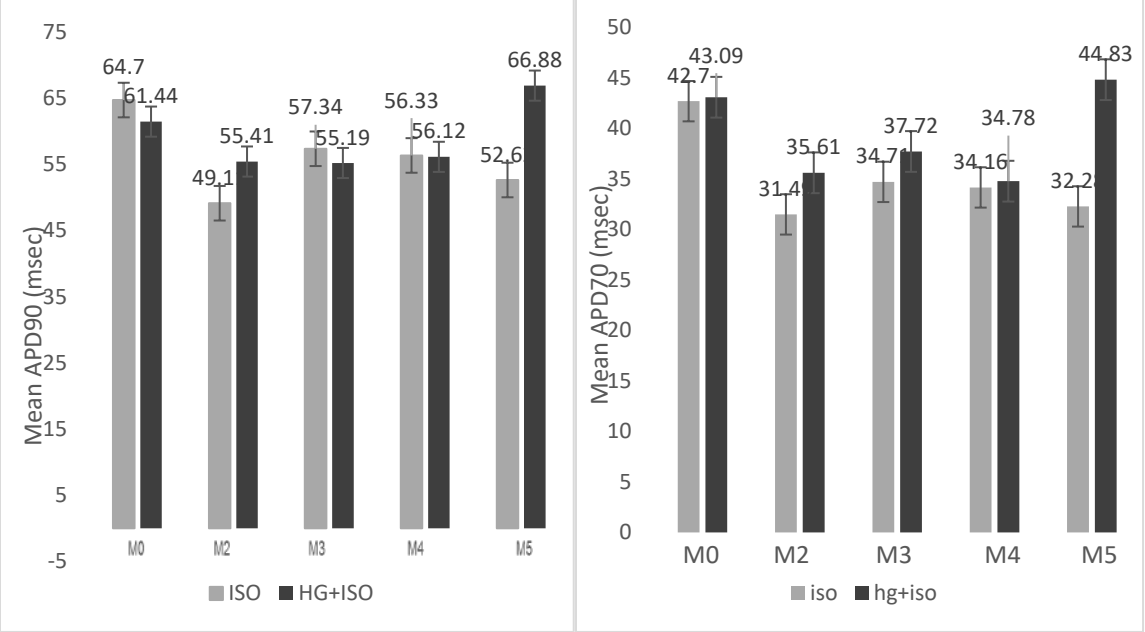
Figure 23: An example of APD restitution curve obtained from Sin20-180 trial. The darker curve represents APD prolongation in the presence of HG compared to ISO (lighter curve). The average increase in APD90 duration in presence of HG was 4.11% (M2).

4.7 c Effect of HG on APD70, APD50 and APD30 compared with ISO using CL- dependent changes

Durations at 90%, 70%, 50% and 30% repolarization were quantified using DP (n=5) and SP (n=4). Animals across these trials showed inconsistent results in presence of HG between the two Cl pacing protocols:

Dynamic protocol results showed APD90 increase in 3 out of 5 animals, and an increase in APD70 in the all 5 animals in presence of HG when added to ISO. When restitution beats were taken (S1-S1 beats) and averaged across the trial for these 5 animals, results showed APD prolongation during DP (see table 8; DP). Below is a bar chart (Figure 24) that shows changes in APD90 and APD70 across the animals.

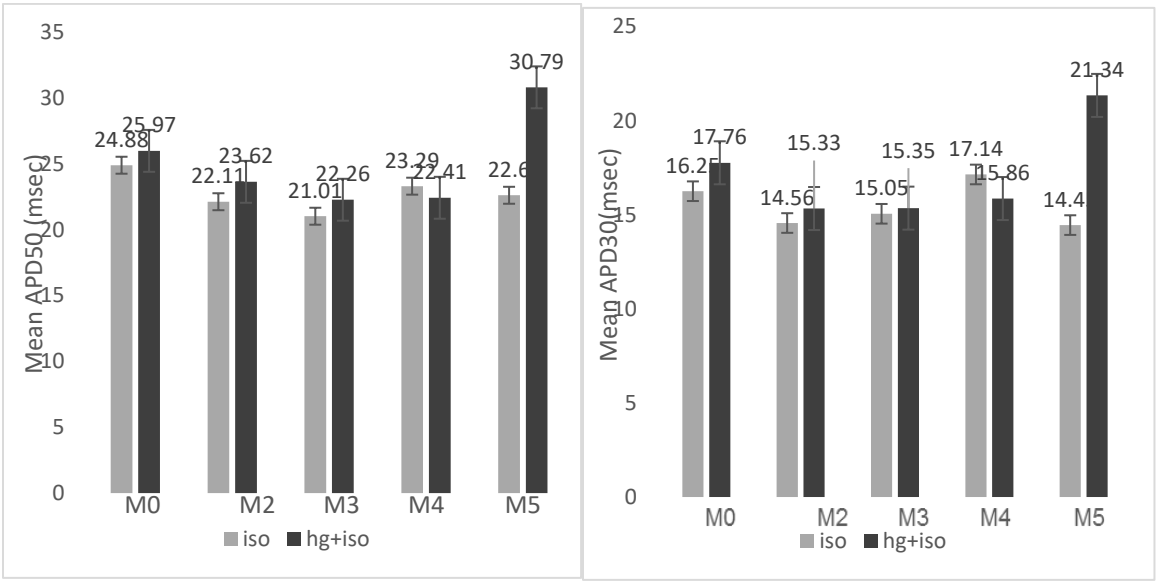
Figure 24.a: (Left-hand side) average APD 90 (msec) (n=5) showed an average increase in 3 animals and decrease in 2 animals in presence of HG added into ISO during DP. Figure 24.b: (Right-hand side) average APD 70 (msec) (n=5) showed an average increase in all 5 animals in presence of HG added into ISO during DP.



Effects of HG after addition to ISO showed an overall increase in APD30 and APD50 durations during DP. In 4 out of 5 animals there was an increase in APD30 and APD50 durations in presence of HG. These durations show that addition of HG by itself increased durations at different stages of repolarization that eventually causes APD

prolongation. Figure 24 c and d are bar charts representing the increase in durations at 30% and 50% repolarization.

Figure 24.c: (Left-hand side) average APD 50 (msec) (n=5) showed an average increase in 4 animals and decrease in 1 animal in presence of HG added to ISO during DP. Figure 24.d: (Right-hand side) average APD 30 (msec) (n=5) showing an average increase in 4 animals and decrease in 1 animal in presence of HG added to ISO during DP.



Results from tissue paced with SP (n=4) were quantified to capture the effect of HG on different levels of repolarization. Results obtained from this data set were inconsistent with the results shown in figure 24.

Specifically, durations at 30% and 50% stages of repolarization during SP showed exactly the opposite change compared to the 30% and 50% durations quantified from the same animals paced during DP. Figure 25 show these changes in duration across different animals

Figure 25.a: (Left-hand side) average APD 90 (msec) (n=4) showed an average increase in 2 animals, decrease in 1 animal and no change in the other during SP. Figure 25.b: (Right-hand side) average APD 70 (msec) (n=4) showed an average increase in 3 animals and decrease in 1 animal in presence of HG when added to ISO during SP.

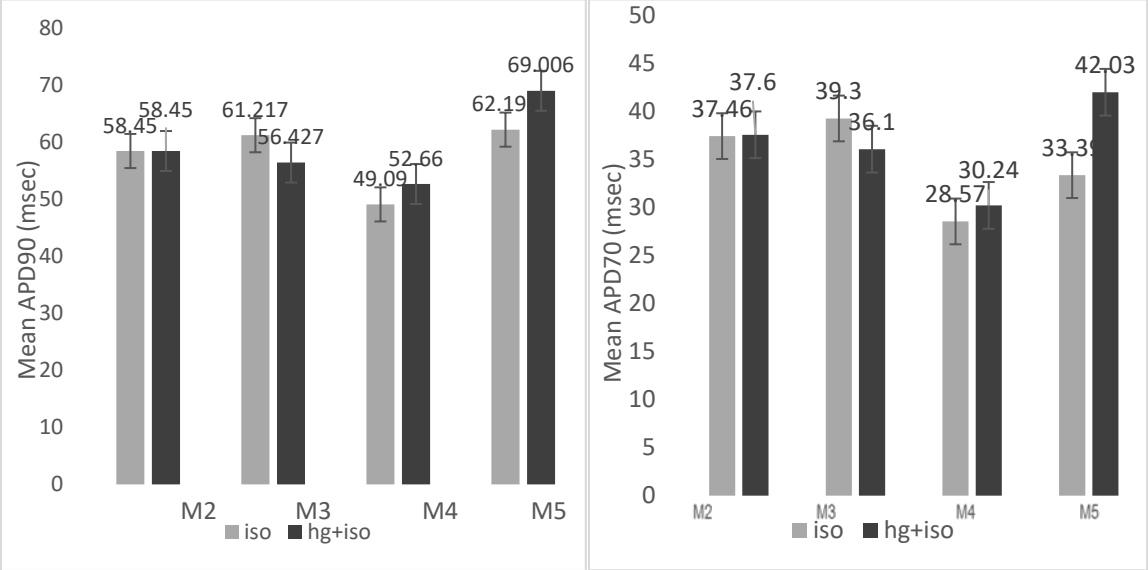
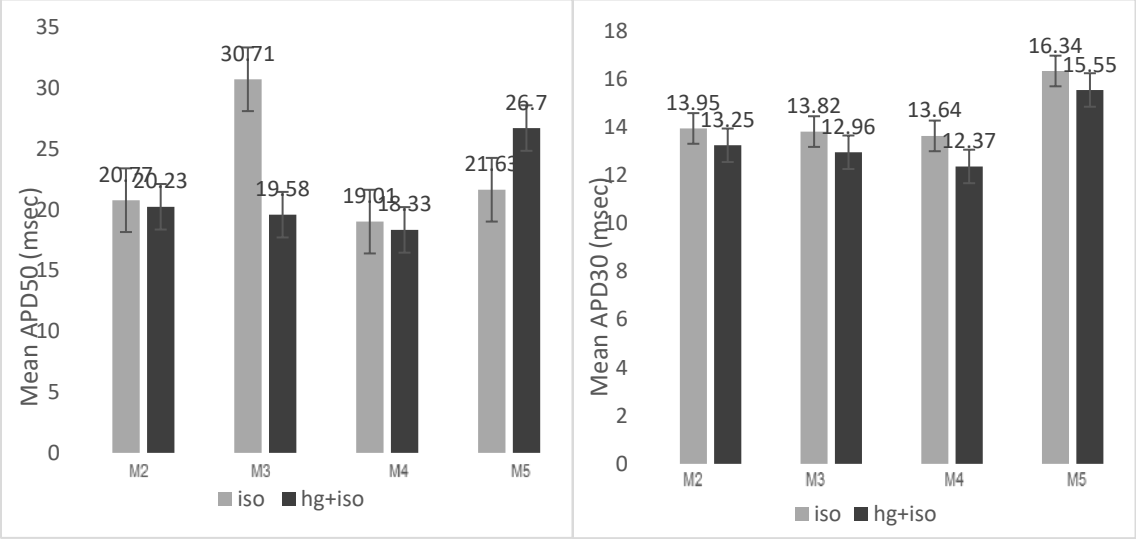


Figure 26.a: (Left-hand side) average APD 50 (msec) (n=5) showed an average decrease in 3 animals and increase in 1 animal in presence of HG added to ISO during DP. Figure 26.b: (Right-hand side) average APD 30 (msec) (n=4) showed an average decrease in 3 animals and increase in 1 animal in presence of HG added to ISO during DP.



4. 7 d Effect of HG on APD70, APD 50 and APD 30 when added to ISO using explicit DI control.

Addition of high glucose to buffer with ISO showed variable results to HG's effect on different stages of repolarization. Repolarization at 70% (n=4) across 4 animals tested at different Sin DI changes from ±80msec to ±50msec showed an overall increase in presence of HG compared to buffer with ISO. This increase in APD70 duration accompanied the increase in action potential duration (APD90).

Early phase action potential durations (APD30 and APD50) were not consistent across animals. For APD50 durations (n=4), animals M2 and M5 showed an increase.

But, animals M3 and M4 showed opposite results. Here, effect of HG on APD 50 gave mixed results and hence not clearly understood. Whereas, APD30 (msec) showed a clear decrease in the duration in presence of HG when compared to ISO in 3 out of 4 animals. An opposite effect can be seen in one animal (M5).

Overall, in presence of HG, APD70 and APD90 increased for the animals and late phase repolarization that is APD 30 showed a clear decrease in 3 animals and increase in 1 animal and APD 50 gave inconsistent results for 2 out of 4 animals with an increase in the other two animals.

4.8 a Effect of HG on action potential amplitude (APA) when added to ISO buffer

Isoproterenol when added to Control buffer by itself increased APA in 2 of 3 animals (see Figure 20). Addition of HG to this tissue further increased the APA. In table 12 are average APA values from Sin DI pacing protocols in 4 animals. 3 out of 4 animals showed an increase in APA after addition of HG to the buffer with ISO when compared to ISO only. During Sin30-170 the results showed from 7% to 18% increase in APA in presence of HG across the 3 animals. The decrease of APA after addition of HG in animal M4 was noticed across all 4 pacing schemes.

Table 22: Average APA values quantified from SIN DI pacing protocols from 4 animals showed an increase of APA in presence of HG for 3 animals and an opposite effect in 1 animal.

Mouse #	Study	Action potential amplitudes (mV)			
		Sin20-180	Sin30-170	Sin40-160	Sin50-150
2	ISO	81.28	84.25	82.21	85.1
	HG+ISO	91.38	90.31	90.87	91.46
	%	11.05 ↑	6.71↑	9.53↑	6.95↑
3	ISO	76.34	82.42	89.64	74.16
	HG+ISO	97.1	100.08	99.78	100.53
	%	21.38↑	17.64↑	10.16↑	26.23↑
4	ISO	91.4	90.01	90.11	86.88
	HG+ISO	74.81	71.61	80.33	92.68
	%	22.17↓	25.69↓	12.17↓	6.25↓
5	ISO	no data	73.54	80.88	82.88
	HG+ISO	85.48	87.39	88.55	87.21
	%		15.84↑	8.66↑	4.95↑

Example of transmembrane potential (Figure 27) below shows the effect of HG on APA compared to ISO buffer. This example was taken from the DP at CL = 120 msec, the average increase in APA during the CL was 11.5 %. When the averages of APA were taken from DP for n=5 animals, 3 animals showed an increase in APA in presence of HG, 1 animal showed no change and 1 animal showed a decreased amplitude with HG.

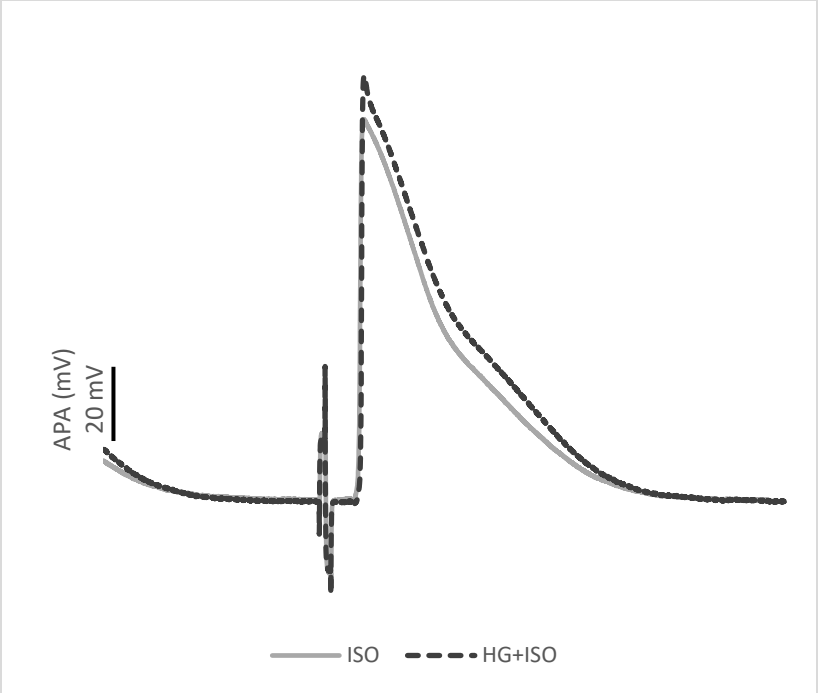


Figure 27: Transmembrane potential showing the effect of HG on APA was to increase the amplitude (dotted) compared to ISO (light solid).

Bar charts (Figure 28) from SP (n=4) showed the same effects as DP (n=3) but, animal M5 that showed an increase in APA in presence of HG during DP, showed an opposite effect during SP. Figure 28 is a bar chart consisting of average APA values for each animal tested with different CL pacing protocols. Dynamic protocol (Figure 28.1) used in n=5 animals showed an average increase in APA for 4 of 5 animals and SP (Figure 28.2) tested on n=4 animals showed an increase of APA in presence of HG in 2 animals and opposite effect in 2 animals.

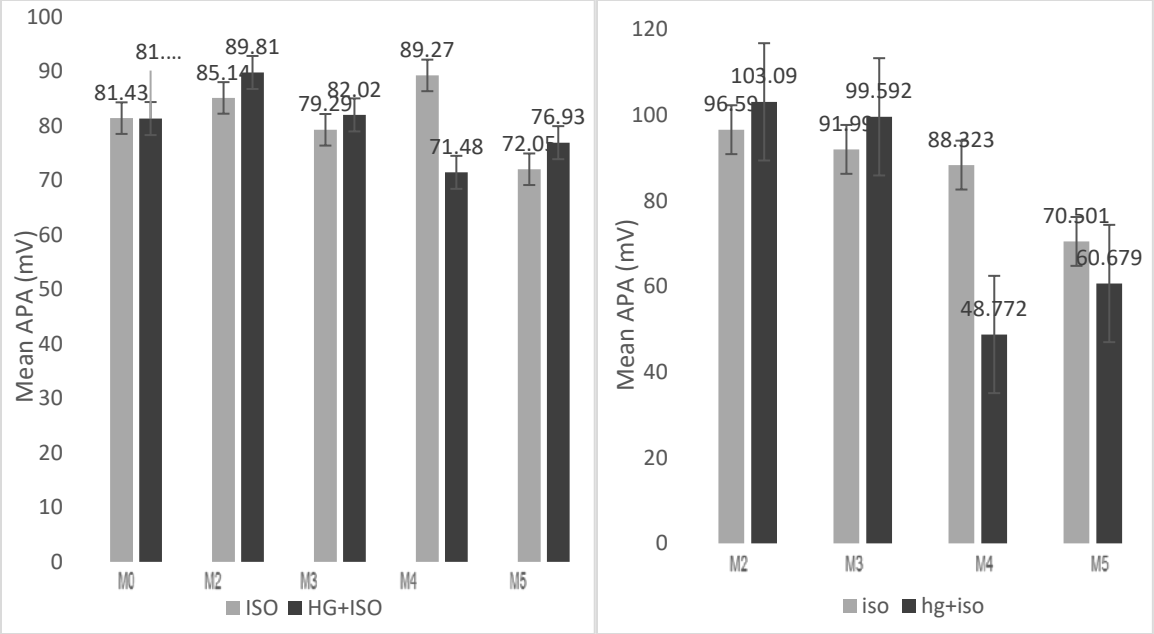


Figure 28.a: (Left-hand side) Average APA values obtained from n=5 animals when tested with DP showed an overall increase of APA in presence of HG in 4 of 5 animals and decrease in 1 animal. Figure 28.b: (Right--hand side) Average APA values obtained from n=4 animals when tested with SP showed an increase of APA in presence of HG in 2 of 4 animals and decrease in 2 animals.

4.8 b Effect of HG on maximum rate of depolarization (dvdtmax) (mV/msec) when added into ISO

Isoproterenol by itself increased the upstroke velocity as shown in part 2, section 4.5 d. When HG was added to the buffer containing ISO, dvdtmax further increased in 3 out of 4 animals in presence of HG and an opposite effect, decrease, was observed in 1 animal (M4). When animal M4 was subjected to ISO only the dvdtmax increased, but with addition of HG to the buffer, the upstroke velocity decreased with a concomitant decrease in APA.

The Table 13 contains observations of dvdtmax (mV/msec) in presence of HG and HG+ISO for tissue tested with Sin DI protocol. The results show an increase in 3 of 4 animals and opposite effect in 1 animal.

Table 23: Average dvdtmax (n=4) in presence of HG after its addition to buffer containing ISO showed an overall increase of dvdtmax.

Mice #	Study	dvdtmax (mV/msec)			
		Sin20-180	Sin30-170	Sin40-160	Sin50-150
2	ISO	28.4	31.65	29.8	33.5
	HG+ISO	50.6	48.9	57.6	59.09
	%	43.87 ↑	35.27 ↑	48.26 ↑	43.3 ↑
3	ISO	15.9	33.03	42.5	11.6
	HG+ISO	53.66	57.06	58.4	58.99
	%	70.36 ↑	42.11 ↑	27.22 ↑	80.33 ↑
4	ISO	57.83	58.53	59.11	51.13
	HG+ISO	34.2	35.5	41.06	45.73
	%	69.09 ↓	64.87 ↓	43.96 ↓	11.8 ↓
5	ISO	18.07	16.07	23.74	25.4
	HG+ISO	27.04	28.97	30.29	29.6
	%	33.17 ↑	44.52 ↑	21.62 ↑	14.18 ↑

This increase of dvdtmax (mV/msec) in presence of HG was also observed when the animals were tested for CL-dependent changes (DP and SP). The increase was observed in 4 out of 5 animals with the opposite effect of HG in 1 animal (M4).

4.9 Effect of HG on alternans when added to ISO

HG by itself showed no influence to alternans when compared to control. After addition of HG to ISO buffer, there were instances of alternans observed across different pacing protocols. All the data showing alternans showed in phase alternans between dvdtmax and APD, APA and APD and dvdtmax and APA. During one of these trials (Animal M3), for only one instance, 4 out of phase alternating beats were observed when tissue was placed in ISO (figure 29). Addition of HG to this buffer then induced in-phase alternans.

4.9 a: Effect of HG on alternans when added to ISO using CL-dependent changes.

Addition of HG to buffer with ISO showed effects of HG on the tissue by inducing alternans at much longer cycle lengths, i.e. slower activation rates than compared to the cycle lengths at which alternans were induced by ISO. For example, when using DP in 4 animals, 2 animals (M4 and M5) clearly showed that addition HG to ISO buffer induced alternans at much slower activation rates in dvdtmax, APA and APD. The other two animals showed an opposite effect after addition of HG to the buffer.

Table 14 contains CL durations at which the first sets of alternans were induced in both ISO and HG +ISO. Below cycle length durations, are the number of alternating beats that were produced. The table shows that in 3 out of 5 animals paced using DP, HG induced alternans at much longer CL's.

Table 24: Cycle length durations at which alternans were present in presence of HG compared to ISO. Durations are collected from DP (n=5) and table shows data from n=3 animals.

Mice #	Study	(In msec)			APA & APD	APA & dvdtmax	dvdtmax & APD
		APA	APD	dvdtmax			
0	ISO	x	110 82 beats	x	x	x	x
	HG+ISO	100 61 beats	120 114 beats	94 110 beats	100 61 beats	94 31 beats	94 65 beats
4	ISO	52 20 beats	56 7 beats	66 51 beats	52 7 beats	52 17 beats	54 4 beats
	HG+ISO	66 16 beats	66 24 beats	70 30 beats	66 9 beats	66 9 beats	66 16 beats
5	ISO	110 221 beats	130 247 beats	120 238 beats	110 220 beats	110 221 beats	120 238 beats
	HG+ISO	120 165 beats	140 181 beats	130 130 beats	120 165 beats	120 119 beats	130 130 beats

The blue highlighted boxes show that HG influenced the tissue by inducing alternans at longer CL's than ISO. From the 3 animals tested, 2 animals show that apart from the CL the number of alternating beats are more in the presence of HG but 1 animal (M5) despite of inducing alternans at much slower activation rates, i.e longer CL, induced fewer alternating beats than ISO.

4.9 b Effect of HG on alternans added to ISO using Sinusoidal DI changes.

Tissues with both ISO and after adding HG were tested using different Sin DI pacing schemes (n=4). From the different pacing schemes, SIN20-180 changes showed most instances of alternans in presence of HG. Table 15 contains data from n=4 animals; 3 animals showed effects of HG on alternans compared to ISO (inducing alternans) and one animal (M5) showed the opposite effect of HG . Addition of HG to the buffer with ISO induced more number of alternating beats of APD in 3 out of 4 animals with an opposite effect on one animal (Animal M5). Animal M3 showed out- of- phase alternans for 4 beats in presence of ISO but after addition of HG in phase alternans are present for 8 beats.

Table 25: Number of alternating beats produced in 4 animals in presence of HG compared with ISO. Blue boxes show the effect of HG on tissue by inducing more number of alternating beats, red depicts for the opposite effect of HG on tissue and X means no alternans observed.

Mice #	Study	no. of beats (Sin 20-180)=240			APA & APD	APA & dvdtmax	dvdtmax & APD
		APA	APD	dvdtmax			
2	ISO	x	x	X	x	x	x
	HG+ISO	x	17 beats	24beats	x	x	7 beats
3	ISO	x	5 beats	6 beats	x	x	4 beats Out of phase
	HG+ISO	4 beats	9 beats	8 beats	3 beats	4 beats	8 beats
4	ISO	x	5 beats	x	x	x	x
	HG+ISO	x	21 beats	x	x	x	x
5	ISO	23 beats	70 beats	31 beats	18 beats	18 beats	28 beats
	HG+ISO	x	50 beats	12 beats	x	x	6 beats

The alternans observed in Sin DI protocol were all in-phase alternans and an example of this is below (figure 29). This example of in-phase alternans is between dvdtmax and APD from animal M3, during sin 20-180 in presence of HG and its comparison to the alternans from ISO only. Figure 29 shows out of phase alternans for 4 beats in presence of ISO and figure 30 shows in phase alternans for 8 beats in presence of HG.

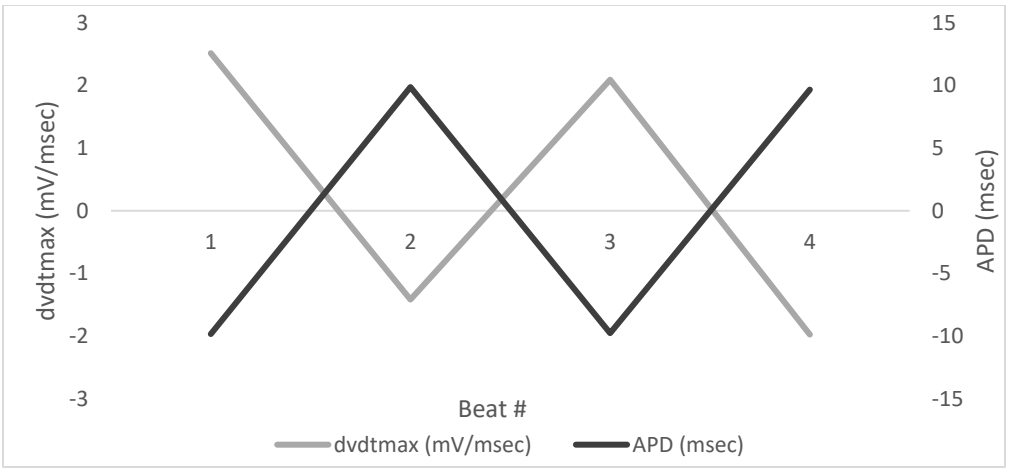


Figure 29: Out of phase alternans between dvdtmax (mV/msec) and APD (msec) for 4 beats in presence of ISO during SIN 20-180.

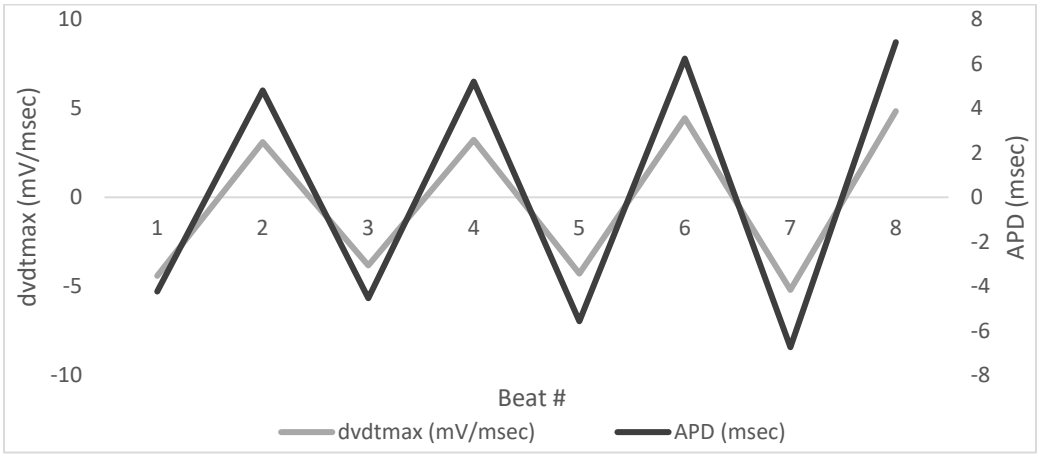


Figure 30: In phase alternans between dvdtmax (mV/msec) and APD (msec) for 8 beats in presence of HG+ISO during SIN 20-180.

4.9 c. Effect of HG when added to ISO on alternans at different levels of repolarization stages (70%, 50% and 30%)

In sinusoidal DI pacing protocols, when HG by itself had some effect on alternans at 70%, 50% and 30% repolarization, once added to buffer containing ISO showed no alternans in either of the stages.

Summary from Part III

Finally addition of HG to a buffer with ISO (HG+ISO) prolonged the APD ever further when compared to results from ISO only. Moreover, when ISO increased the dvdtmax and APA by itself, addition of HG to ISO buffer further increased the dvdtmax and APA values. Apart from APD increase, an increase in APD70 was also observed.

HG + ISO resulted in alternans but this was not the predominant effect. Most instances of alternans were observed during DP where, in 3 out 5 animals addition of HG to ISO buffer had a greater alternans threshold and increased number of alternating beats when compared to results from ISO only.

Chapter 5: Conclusions

Diabetes is considered a major risk factor for cardiovascular diseases. One mechanism by which this risk is increased is through its effects on electrical disturbances within the heart leading to ventricular arrhythmias and possibly sudden cardiac death (SCD). Hyperglycemia is known to increase the action potential duration (APD) in rat myocytes [24] decreasing the refractoriness of the cell. We have demonstrated that acute changes in glucose levels has similar effects.

The motivation underlying this project was that acute exposure to elevated glucose, i.e. acute hyperglycemia (HG), through either calmodulin kinase II or other, as yet unknown pathways, would modify electrophysiological function of cardiac myocytes and, if that is the case, then acute HG would also alter the adrenergic mediated changes in electrophysiological function.

5.1 Action potential duration features

Our results showed that when the tissue was subjected to high glucose buffer solutions, APD increased. This observation was seen in presence of acute exposure to high glucose in 4 animals with the increase in APD ranging from 14% to 25% when compared to Control. Nobe S et al showed a significant increase in action potential duration in diabetic rat ventricular tissues with an altered Ca2+ current. Shigametsu S [34] also showed APD prolongation in diabetic rat myocytes at different levels of repolarization (APD 25, APD 50, APD75 and APD90) with an increase in APD prolongation compared to normal myocytes. Additionally, studies by Kathryn et al [35] showed an increase in AP duration in STZ-treated animal myocytes at 20%, 50% and

90% repolarization stages. Similar to this study, our results also showed that APD90 prolongation was accompanied by an overall increase in APD70 but, the early phase repolarization (APD30 and APD50) showed an increase only in 1 animal and decrease in the others. Taken together, our results suggest that HG by itself can cause APD prolongation. Apart from the changes due to addition of HG where, APD70 and APD90 duration increased in, HG had more or less minimal effect on the early phase repolarization because consistent changes to the early phases of repolarization were not seen. Bers et al, showed in their studies that addition of acute HG activated CaMKII protein kinase and caused both early and late phase prolongation, therefore the results from our study are somewhat inconsistent with their results as far as early phase repolarization durations are concerned.

Adrenergic effect in the tissue was also altered in the presence of HG. While increasing adrenergic stimulation using Isoproterenol decreased APD, addition of HG prolonged APD in 3 out of 4 animals with an opposite effect seen in an animal. Effect of HG on APD70 during increased adrenergic stimulation showed an overall increase in APD70. However, with HG and increased adrenergic stimulation, early phase repolarization stages showed an overall decrease at 30% repolarization in 3 out of 4 animals during certain stimulation protocols (Sinusoidal DI changes) but different changes during other protocols (CL- dependent changes). Similarly, changes in APD50 were also not consistent. Further, the changes observed in APD30 and APD50 during DP were opposite to the APD30 and APD50 analyzed during SP. The reasons for this disparity are not completely clear, but, given that rodents such as mice do not display the characteristic plateau that is calcium dependent, it is possible that camKII

modulated effects manifest in later stages of the AP. However, it is also a possibility that acute HG may have an effect on potassium dependent repolarization currents, studies using selective blocking agents for these currents would be required to explore these effects. If the modifying effect of HG on adrenergic stimulation would have been via changes in calcium handling as would have been predicted, then we would have expected to observe consistent changes in early repolarization durations, i.e. in APD30 and APD50. Therefore, based on our results, it remains unclear as to how acute increases in glucose levels modify the adrenergic effects on action potential repolarization.

5.2 APA and dv/dt max phase behavior

The maximum upstroke velocity, i.e. maximum rate of depolarization is governed by the opening of Na+ channels allowing the influx of Na+ ions. More the sodium channels open, more is the influx and faster is the upstroke velocity.

Generally a slow upstroke by itself does not affect DI, rather its effect on conduction has the largest effect. But those effects are difficult to predict unless one knows about conduction properties between cells. Something that causes a slow upstroke can also cause APD to be shorter (in general) which at constant CL can make DI longer, but again it is difficult to predict which way the effect will be.

In the literature, there are mixed results about the effects of HG and its modification of upstroke velocity. Observations by Kathryn et al., showed that depolarization rate decreases significantly in presence of high glucose (HG) as compared to Control. In our study, when compared to Control, during Sinusoidal DI and DP (n=3), rate of

depolarization decreased in 2 out of 3 animals with an opposite effect in 1 animal. For SP, (n=4) the rate of depolarization increased in 2 animals and decreased in 2 animals.

Therefore, our results do not show changes in rate of depolarization that are consistent between animals and with previously reported changes.

Effect of HG on action potential amplitude also varied between Control and ISO buffer. When compared to Control, during Sinusoidal DI and DP (n=3), action potential amplitude decreased in 2 out of 3 animals with an opposite effect in 1 animal. For SP, (n=4) the action potential amplitude increased in 2 animals and decreased in 2 animals. These results show the same pattern of increase/decrease as was the case with maximum rate of depolarization in the same animals. With adrenergic activity, presence of HG caused an overall increase in the APA across different pacing control schemes. For n=5 animals when ISO was compared to HG + ISO, majority of the animals showed a consistent increase in APA in presence of HG. It is peculiar that APA showed consistent increase while dvdtmax did not, as in many cases APA and dvdtmax are related. The reasons for this de-coupling are not clear, although an intriguing, but speculative, reason may be effects of HG on cell to cell coupling reduce cellular electrical load which can produce larger APA even when dvdtmax remains the same or even decreases. Further exploration in studies that permit exploration of conduction velocity would be necessary to determine if such effects may have contributed to the observed increase.

5.3 Alternans

For CL pacing protocols, when the tissue showed alternans in Control, addition of HG decreased the number of alternating beats.

When HG was compared when the adrenergic level was high (ISO), there were alternans seen when tissue was paced with Sin DI and CL pacing protocols. For Sin DI protocols (n=4) 3 animals showed incidences when HG influenced alternans by increasing the number of alternating beats when compared to ISO only. One animal showed an opposite effect when the tissue was more stable in presence of HG (generally, less number of alternans or alternans occurring at higher rates is considered to increase stability). In CL pacing protocols (n=5), 3 animals clearly show that when HG was added to the tissue, alternans were induced at much slower activation rates (longer cycle lengths) and in 2 animals, addition of HG to buffer with ISO decreased the number of alternating beats.

In conclusion, our results suggest that HG does modify electrophysiological features of action potentials in a murine model and importantly, HG also seems to alter the effect of adrenergic stimulation. However, the effects of HG on electrophysiology and on adrenergic effects are not consistent among animals. Given the clinical observation that elevated glucose also has differing effects in terms of cardiovascular morbidity, these results support further exploration of effects of acute changes in glucose on cardiac electrophysiology as it relates to changes in risk of arrhythmic events.

Chapter 6: Limitations

We were not able to record time control data because of tissue viability. To keep the tissue alive for long periods of time was a challenge and we were not able to compare time control data with other data sets which were acquired during protocols. Acquiring time control data would have been helpful to better understand effect of time on APD.

All 6 mice used during the experiments were female. Since it was anticipated early on in the study that the number of animals from which we would obtain useful data was going to be low, having all mice of the same sex was advantageous as it did not add sex as an additional confounding variable. Future studies will be necessary to determine whether sex affects these effects of HG. However, the estrous status of the used mice was unknown and therefore is a factor that could have affected some of the inter-animal variability that we observed.

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